University of Alberta

Investigating post-translational control of BldD in *Streptomyces coelicolor*

by

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Abstract

The DNA-binding transcriptional regulator, BldD, may function as both a repressor and an activator of developmental and antibiotic synthesis genes in *Streptomyces coelicolor*. Specific modification of the BldD protein itself, or the interaction of BldD with a binding partner, are two of the possibilities for how BldD activity might be modulated. A number of biochemical techniques were employed to address these two possibilities. Although these methods did not lead to characterization of a modified BldD protein or a BldD-binding partner, two-dimensional separation of *S. coelicolor* cell-free extract proteins identified at least three putative modified BldD isoforms and chemical crosslinking studies identified a possible BldD protein complex. Further support for the activator function of BldD was obtained by chromatin immunoprecipitation analyses, which indicated that BldD could bind *in vivo* to the *sigQ* promoter, a putative activated target of BldD. Semi-quantitative RT-PCR results suggested that *sigQ* expression is activated by BldD.

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List of Abbreviations

α	Alpha
Δ	Delta or deletion
ε	Epsilon
γ	Gamma
λ	Lambda bacteriophage
μ-	Micro-
ϕ	Phi
σ	Sigma factor
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
3D	Three-dimensional
A	Adenine
ATP	Adenosine triphosphate
bp	Base pair(s)
BS^3	Bis(sulfosuccinimidyl)suberate
BSA	Bovine serum albumin
C	Cytosine (DNA) or Cysteine (Amino acid)
C-	Carboxy-
°C	Degrees Celcius
cat	Chloramphenicol resistance gene
cDNA	Complementary DNA
CHAPS	3-[(-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate
ChIP	Chromatin immunoprecipitation
cm	Centimetre(s)
CNBr	Cyanogen bromide
cpm	Counts per minute
Da	Dalton(s)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DMP	Dimethyl pimelimidate·2HCl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA (agar)	Difco nutrient agar
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate

ECF	Extracytoplasmic function
EDTA	Ethylenediamenetetraacetic acid
EGS	Ethylene glycolbis(succinimidylsuccinate)
g	Gram(s)
G	Guanine
HEPES	Hydroxyethylpiperazinethansulfonic acid
His	Histidine
HTH	Helix-turn-helix
IAA	Isoamyl alcohol
IgG	Immunoglobulin G
IP	Immunoprecipitation
IPTG	Isopropyl β-D-thiogalactopyranoside
ISP4	International *Streptomyces* Project medium 4
kan	Kanamycin resistance gene
kb	Kilobase(s)
kDa	Kilodalton(s)
L	Litre(s)
LB	Luria-Bertani medium
LC MS/MS	Liquid chromatography tandem mass spectrometry
M	Molar
mA	Milliamp(s)
Mb	Megabase(s)
MBSU	Molecular Biology Service Unit
mg	Milligram(s)
mL	Millilitre(s)
mm	Millimetre(s)
mM	MilliMolar
mQH_2O	MilliQ water
MS medium	Mannitol soy medium
MWCO	Molecular weight cut off
MWM	Molecular weight marker
m/z	Mass to charge ratio
N-	Amino-
NaOAc	Sodium acetate
Ni-NTA	Nickel-Nitrilo-tri-acetic acid
nm	Nanometre(s)
nM	NanoMolar
NMMP	Minimal liquid medium
NP-40	Nonidet P40

nt	Nucleotide(s)
NTP	Nucleoside triphosphate
OD	Optical density
ORF	Open reading frame
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFAM	Protein family
pI	Isoelectric point
pmoles	Picomoles
R2YE	Sucrose yeast extract medium
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMM	Supplemented minimal medium
SMMS	Supplemented minimal medium, solid
spp.	Species
SSIII	SuperScript™ III
SSC	Standard saline citrate
T	Thymine
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	Tetramethyl ethylene diamine
T_m	Melting temperature
TSB	Trypticase soy broth
tsp	Transcription start point
tsr	Thiostrepton resistance gene
Tyr	Tyrosine
UV	Ultraviolet
V	Volts
v/v	Volume per volume

w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indoyl–β-D-galactopyranoside
XRE	Xenobiotic response element
Y	Tyrosine
YEME	Yeast extract-malt extract medium
YT	Yeast extract tryptone

CHAPTER 1:

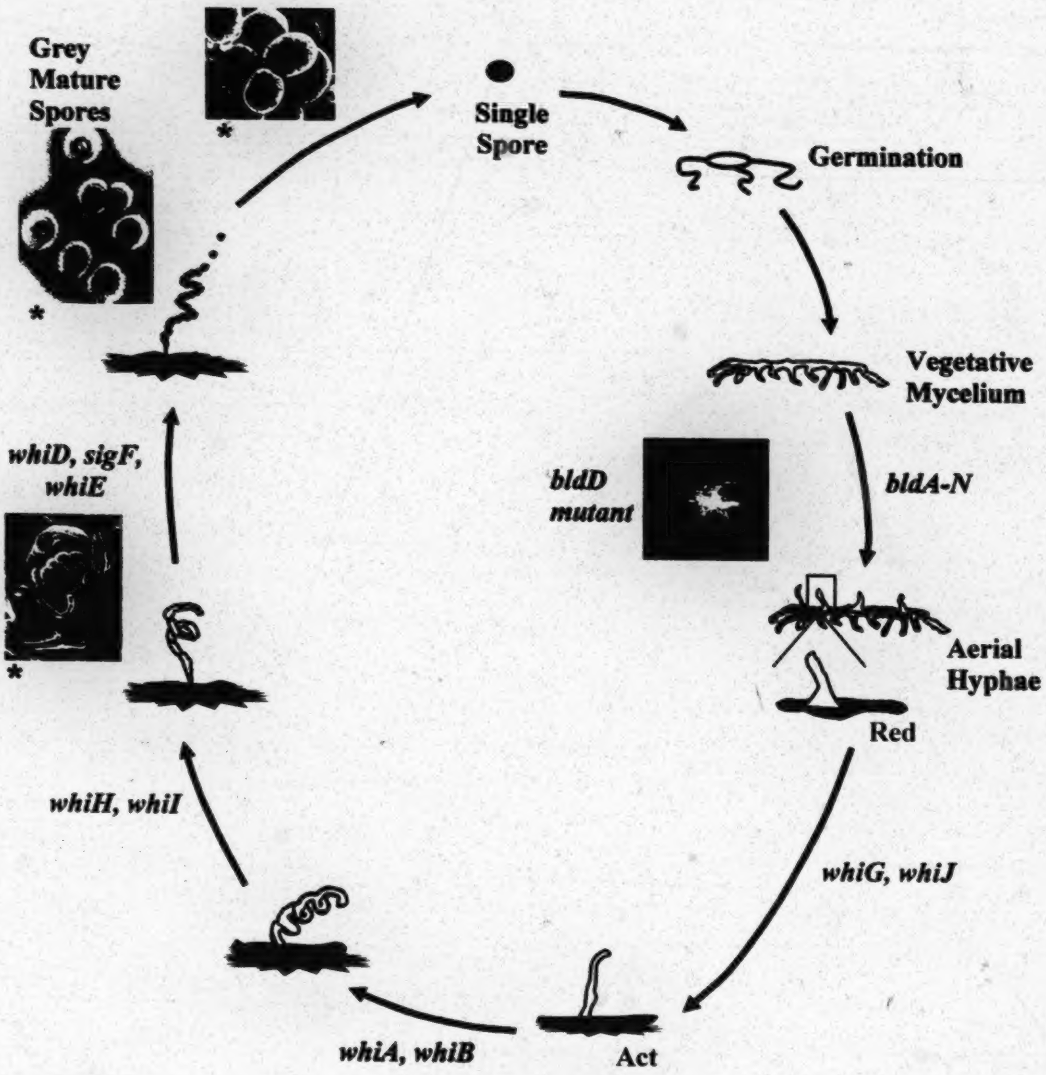
INTRODUCTION

1. INTRODUCTION

1.1. The genus *Streptomyces*

1.1.1. Complex life cycle

Streptomyces spp. are Gram-positive, soil-dwelling bacteria subject to much intense research because they possess a developmental program that is very complex by prokaryotic standards. These multicellular bacteria have a fascinating life cycle characterized by several morphologically differentiated states (Figure 1.1). The life cycle begins with the germination of a single spore to produce a vegetative mycelium, a mat of branching hyphae in which numerous adjacent genomes are separated by few crosswalls. To obtain nutrients for growth, the vegetative mycelium releases extracellular hydrolytic enzymes to solubilize organic debris found in the soil (Chater 1984). In response to an as-yet unknown signal(s), perhaps arising from nutritional limitation or environmental stress, the vegetative mycelium ceases growth and differentiates to give rise to the aerial mycelium, a network of hyphae that grow into the air. It is thought that nutrients for the growth of aerial hyphae originate from vegetative hyphae that die through a process of orderly cell dismantling resembling programmed cell death seen in animal cells (Miguelez et al. 1999). Degradation of intracellular glycogen storage reserves creates turgor pressure, another important factor allowing for successful erection of aerial hyphae into the air (Chater 1989; Plaskitt and Chater 1995). Further morphogenesis of aerial structures occurs through a process of coiling and regular septation to produce long chains of unigenomic spores that are eventually released into the environment for dispersal to favourable growth conditions, thus allowing the growth cycle to continue.


Single Spore
Germination
Vegetative Mycelium
bldA-N
bldD mutant
Aerial Hyphae
Red
whiG, whiJ
Act
whiA, whiB
whiH, whiI
whiD, sigF, whiE
*
Grey Mature Spores
*
*

*Photos courtesy of The Sanger Institute: *S. coelicolor* genome project

1.1.2. Commercial relevance

Besides their morphological complexity, the streptomycetes are famous as producers of a wide variety of medically and industrially important secondary metabolites, many of which are antibiotics. Interestingly, the production of these important secondary metabolites occurs at the time when aerial growth commences, suggesting that control of morphological differentiation and secondary metabolism share some common regulatory elements (Chater 1984). Information gained from studies focused on unraveling the complex regulatory pathways directing morphological differentiation could potentially shed light on the pathways controlling antibiotic synthesis. This knowledge could therefore provide insight into how antibiotic biosynthetic pathways might be altered to improve fermentation efficiency, either of naturally produced or of new hybrid antibiotics.

1.1.3. *Streptomyces coelicolor* A3(2): a model streptomycete

Streptomyces coelicolor A3(2) is the most-studied and genetically best-characterized representative of this genus. It is the model organism for studying morphological differentiation and antibiotic biosynthesis in the streptomycetes. Three main factors have contributed to the rapid advancement of *S. coelicolor* research. First, *S. coelicolor* produces two pigmented antibiotics, allowing for an effective visual screen of mutants defective in antibiotic production (Champness 2000). Second, antibiotics produced by this organism are not clinically relevant so information is more readily shared among labs. Third, the completion of the *S. coelicolor* genome sequence has provided useful annotative information for study in this bacterium (http://www.sanger.ac.uk/projects/S_coelicolor). Furthermore, the completion of the genome sequence has facilitated the use of microarray analysis to examine global regulation of gene expression, thus allowing for the identification of novel genes involved in either differentiation or secondary metabolism.

S. coelicolor possesses a large 8.67 Mb chromosome of approximately 72% G + C content (Bentley et al. 2002). The genome of this organism consists of 7825 putative coding sequences, and 12.3% of these genes encode regulatory proteins including abundant two-component regulatory systems and 65 sigma factors (the highest number found in a bacterium to date). In addition, there are 22 gene clusters for secondary metabolism, and three of these clusters are specifically involved in antibiotic production (Bentley et al. 2002). A fascinating feature of the *S. coelicolor* chromosome is that it exists in a linear form, rather than in the circular form expected for a prokaryotic organism (Lin et al. 1993). Furthermore, *S. coelicolor* harbours three plasmids including SCP1, a large 365 kb linear plasmid (Kinashi and Shimaji-Murayama 1991); SCP2, a 31 kb circular plasmid (Bibb et al. 1977); and SLP1, a 17 kb plasmid capable of site-specific integration into the chromosome (Omer and Cohen 1984). These three plasmids serve as fertility factors and are useful genetic elements for DNA cloning.

1.2. Regulation of morphological differentiation

1.2.1. Genes controlling sporulation

1.2.1.1. The *whi* genes

The *whi* genes are dispensable for vegetative growth and aerial mycelium formation, but are required for morphogenesis of aerial hyphae into spore chains (Hopwood et al. 1970). *whi* mutants are defective in spore formation, giving a white colony appearance due to the lack of mature, grey-pigmented spores in the aerial hyphae

(Hopwood et al. 1970). Fifty white mutants were isolated in the first screen for spore-defective mutants, and were mapped to eight distinct loci designated *whiA-I* (Chater 1972; Hopwood et al. 1970). *whiC* and *whiF* are no longer included because the former could not be recovered from lyophils and the latter was found to be an allele of *whiG* (Ryding et al. 1999). Two other mutants, isolated in the original screen and appearing to have the same phenotype as the *whiC* mutant, were later named *whiJ* because the relationship between these two mutants and the *whiC* mutant could not be defined (Chater 1998). *whiJ* has been proposed to act early in the sporulation cascade, although little is known of how *whiJ* exerts its control on sporulation processes (Chater 1998). A second screen for mutants deficient in spore formation identified five new sporulation loci designated *whiK-O* (Ryding et al. 1999). These genes are mostly uncharacterized except for *whiK* and *whiN* which were later found to be novel *bld* loci because null mutations in these genes eliminated aerial mycelium formation (Bibb et al. 2000; Molle and Buttner 2000). The *whi* genes are further classified into two groups of early and late *whi* genes. The early *whi* genes (*whiA*, *B*, *G*, *H*, *I*, *J*) are needed to control the initial stages of spore formation, including aerial hyphae coiling and septation. Conversely, the late *whi* genes (*whiD*, *sigF*, *whiE*) are required for spore maturation and pigmentation. Based on the sporulation stage that is blocked in the *whi* mutants, the *whi* genes are proposed to act in the following order: *whiG*, *whiJ* > *whiA*, *whiB* > *whiH* > *whiI* > *whiD*, *sigF*, *whiE* (Chater 1998).

A *whiG* mutant produces straight, aseptate aerial hyphae, while overexpression of *whiG* results in hypersporulation in the aerial hyphae and ectopic sporulation in the vegetative hyphae (Chater 1972; Chater et al. 1989; Mendez and Chater 1987). These observations, along with the finding that transcription of *whiG* does not depend on any of the six known early *whi* genes, place *whiG* at the top of the hierarchy of *whi* genes and indicate that the *whiG* gene product plays a role in the earliest stages of sporulation (Kelemen et al. 1996). *whiG* encodes an alternative sigma factor most similar to *Bacillus subtilis* σ^D, a sigma factor involved in regulating gene expression for motility, chemotaxis, and autolysin production (Chater et al. 1989; Helmann et al. 1988). Introduction into *S. coelicolor* of a DNA fragment containing a σ^D-dependent promoter results in decreased spore formation, likely due to the sequestration of σ^{WhiG} away from sporulation-specific promoters (Chater et al. 1989). Like other sigma factors, σ^{WhiG} might be regulated by a post-translational mechanism involving interaction with an anti-sigma factor homologue. Lending support to this idea is the finding that during vegetative growth, when σ^{WhiG}-dependent genes are not transcribed, *whiG* transcript levels are comparable to those found during morphogenesis of aerial hyphae into spores (Kelemen et al. 1996). The cognate anti-sigma factor for σ^{WhiG}, however, has not yet been identified.

After *whiG* expression, the next sporulation checkpoint requires the *whiA* and *whiB* genes. Mutants of *whiA* and *whiB* produce long, tightly coiled aerial hyphae with few sporulation septa (Chater 1972). *whiA* encodes a protein of unknown function that is conserved among Gram-positive bacteria (Ainsa et al. 2000). *whiA* transcription is developmentally regulated, with transcripts most abundant in sporulating aerial hyphae. The observation that *whiA* transcript levels are reduced in a *whiA* mutant suggests that the WhiA protein may act as a positive autoregulator. WhiA, however, is not similar to any other known transcriptional regulator and it does not possess any DNA-binding motifs.

Conversely, *whiB* encodes a protein of 87 amino acids proposed to be involved in both DNA binding and interaction with other proteins to promote transcriptional activation of genes controlling sporulation (Davis and Chater 1992). *whiB* transcription is initiated from two promoters where *whiBP1* activity is weak and *whiBP2* activity is developmentally regulated, increasing at the time of aerial mycelium formation (Soliveri et al. 1992). *whiB*-like genes have been identified in other actinomycetes, where they are known as the *whm* genes (*whiB* homologues in mycobacteria) in *Mycobacterium* spp. and the *wbl* genes (*whiB*-like) in other streptomycetes (Soliveri et al. 2000). An alignment of these WhiB-like proteins suggested that they have DNA-binding capabilities, and that they too might function as transcriptional regulators. In addition, these proteins contain four conserved cysteine residues, suggesting that they may function in sensing redox changes that would presumably occur during environmental stresses or nutritional shift-down (Soliveri et al. 2000). Since *whiA* and *whiB* mutants produce unusually long aerial hyphae, it is believed that the *whiA* and *whiB* gene products are involved in the cessation of aerial hyphal growth, thus allowing the process of spore septation to proceed (Flardh et al. 1999).

Based on the observations that the aerial hyphae of *whiH* mutants are not unusually long, that *whiG*, *whiA*, and *whiB* are epistatic to *whiH*, and that genes involved in the later stages of sporulation are transcribed at low levels in a *whiH* mutant, *whiH* is probably the third decision point in the sporulation cascade (*whiG* > *whiA*, *whiB* > *whiH*) (Flardh et al. 1999). In contrast to *whiA* and *whiB*, transcription of *whiH* is directly dependent on the *whiG* product (Ryding et al. 1998). The product of the *whiH* gene contains a helix-turn-helix DNA-binding domain and resembles regulatory proteins belonging to the GntR family of regulators. Since *whiH* mutants are defective in sporulation, WhiH may function as a positive regulator of sporulation. However, because other known GntR regulatory proteins function as repressors, it is proposed that WhiH may instead act as a repressor of negative regulators of sporulation. WhiH seems to exhibit autorepression, and transcription of *whiH* is developmentally regulated with expression peaking during spore formation. It is proposed that for WhiH, as for several other GntR-like regulators from *Escherichia coli*, repression of both *whiH* transcription and target gene expression is relieved when WhiH interacts with a particular metabolite, whose concentration varies during growth (Ryding et al. 1998).

The last early *whi* gene in the sporulation cascade is *whiI*. *whiI* mutants have aerial hyphae similar to those of *whiH* mutants, but resemble coiled fragments due to occasional sporulation septa (Chater 1972). *whiI* codes for an atypical response regulator but has not been associated with a sensor kinase (Ainsa et al. 1999). As is the case with *whiH*, transcription of *whiI* depends directly on the *whiG* product. Furthermore, it is thought that WhiI activity, like WhiH activity, might be modified by binding to a specific metabolite, whose concentration would change during development. In addition, WhiH and WhiI seem to regulate the expression of one another, meaning that derepression of *whiH* expression occurs in a *whiI* mutant, and *whiI* expression is slightly affected in a *whiH* mutant. It is postulated that WhiH and WhiI might function to activate transcription of the late *whi* genes, thus providing a developmental link between the early and late *whi* genes (Ainsa et al. 1999; Chater 2001).

whiD and *whiE*, along with *sigF*, are required for the later stages of spore formation, following the production of immature, unpigmented spores. The *whiD* product, which is a homologue of WhiB, is believed to be a putative transcriptional regulator (Molle et al. 2000). WhiD is capable of binding a [4Fe-4S] cluster suggesting that its role in differentiation might involve sensing redox changes that coincide with alterations in cellular metabolism (Jakimowicz et al. 2005). The transcription of *whiD* is under the control of two developmentally regulated promoters that remain inactive until the onset of sporulation (Molle et al. 2000). A *whiD* mutant produces wild-type levels of spores, but these are unpigmented, thin-walled, and lyse frequently (Chater 1972; McVittie 1974). Additionally, *whiD* null mutants contain irregular, small, chromosome-free compartments within individual spores resulting from septa formation along different planes (Molle et al. 2000). These observations indicate that the *whiD* product controls spore maturation and likely plays an important role in spore wall formation.

The *sigF* locus, although not named for the white colony phenotype conferred by mutants of this gene, is required for normal sporulation. The *sigF* locus encodes a sigma factor most similar to the general stress response σ^B of *B. subtilis*, but does not share similar functions with σ^B (Boylan et al. 1993; Potuckova et al. 1995). A *sigF* mutant produces thin-walled, unpigmented spores containing uncondensed DNA, indicating that σ^F is required for expression of genes in the late stages of sporulation (Potuckova et al. 1995). *sigF* transcription is dependent on all six early *whi* genes and is temporally correlated with septum formation, indicating that *sigF* expression is spatially localized to spore compartments (Kelemen et al. 1996; Sun et al. 1999). Transcription of *sigF* is dependent on *whiG*, but this dependence is likely indirect since the *sigF* promoter is not similar to other known σ^{WhiG}-dependent promoters (Kelemen et al. 1996). It is possible that another sporulation-specific sigma factor lying between σ^{WhiG} and σ^F is responsible for directing transcription of the latter sigma factor gene (Kelemen et al. 1996). Alternatively, it is possible that under certain conditions *sigF* may be transcribed by RNA polymerase holoenzyme containing the principle housekeeping sigma factor HrdB (Homerova et al. 2000). This hypothesis is based on the finding that two DNA-binding proteins that control *sigF* expression in *Streptomyces aureofaciens* have been identified, and it is proposed that RsfA (regulator of *sigF*) may act as a repressor during vegetative growth whereas RsfB may act as an activator during sporulation. It is therefore plausible that the *sigF* promoter might be recognized by HrdB-bound RNA polymerase when an activator, perhaps RsfB, is bound to the *sigF* promoter.

The final stages of the sporulation cascade involve *whiE*. *whiE* mutants produce spores exhibiting wild-type morphology but lacking the characteristic grey pigmentation (Chater 1972; McVittie 1974). The *whiE* locus encodes seven open reading frames (ORFs) with six of these genes sharing high homology with genes for the synthesis of polyketide antibiotics (Davis and Chater 1990). This gene cluster, which was later found to include at least one other divergently transcribed gene, is required for the production of the grey spore pigment (Davis and Chater 1990; Kelemen et al. 1998). Transcription of the *whiE* cluster is under the control of two divergent promoters, both of which are transiently expressed at the time of sporulation and are dependent on all six early *whi* genes, indicating that pigment production is confined to spore compartments (Kelemen et al. 1998). While *whiEP1* activity is not dependent on the product of *sigF*, *whiEP2* transcription shows *sigF* dependence. However, *whiEP2* could not be transcribed by σ^F under *in vitro* conditions, suggesting that an activator might be required for transcription in the normal situation.

Evidently, the control of spore formation involves an intricate regulatory network rather than a simple linear cascade. Studies of the *whi* genes mentioned above have provided much insight into how aerial hyphae further differentiate into spore chains. However, the observation that many of the *whi* loci were represented by only one or two mutations in the original screens, likely means that other *whi* genes remain to be discovered (Chater 1972).

1.2.1.2. *ftsZ* and the *ssg* genes

In addition to the *whi* genes, other genes involved in the sporulation process have been identified. Whereas *ftsZ* is essential for viability in the unicellular bacterium *E. coli* (Dai and Lutkenhaus 1991), in *S. coelicolor*, this gene is dispensable for vegetative growth and for proper aerial mycelium formation, but is obligately required for sporulation (McCormick et al. 1994). Interestingly, the processes of spore formation and spore pigment production are uncoupled in an *ftsZ* mutant, suggesting that this mutant might define a new group of developmental mutants separate from the *whi* mutants, which are blocked in both spore and pigment production (McCormick et al. 1994). Long, orderly ladders of FtsZ rings are found in wild-type *S. coelicolor* aerial hyphae, but are absent from those of *whiB*, *whiG*, and *whiH* sporulation-defective mutants (Schwedock et al. 1997). It is not known, however, whether *ftsZ* expression is directly or indirectly dependent on these *whi* genes. Further characterization of *ftsZ* identified three putative promoters for transcription of this gene (Flardh et al. 2000). One of these promoters, *ftsZ2p*, which is regulated developmentally and under the control of the six early *whi* genes, is not required for vegetative growth but is needed for sporulation. It is still not known how the early *whi* genes exert control on *ftsZ2p* activity, although it is likely that transcription from this promoter is not directly dependent on σ^{WhiG} because this promoter does not resemble the consensus of other known σ^{WhiG}-dependent promoters.

Seven genes, *ssgA-ssgG* (sporulation specific cell division gene), comprise a new family of paralogous genes encoding proteins implicated in sporulation control (Keijser et al. 2003). Of these genes, only *ssgA* and *ssgB* have been characterized. *ssgA* was first identified in *Streptomyces griseus* and was proposed to play a role in sporulation and cell division because SsgA accumulates at the onset of sporulation but does not accumulate in certain developmental mutants (Kawamoto et al. 1997). Overexpression of SsgA results in fragmented growth, which indicates that this protein normally functions in septum formation (Kawamoto et al. 1997). An *ssgA* null mutant in *S. coelicolor* has a white colony phenotype, providing further support that this gene is a novel *whi* gene required for proper sporulation (van Wezel et al. 2000). Although an *ssgA* mutant cannot produce spores, aerial hyphae in this mutant exhibit the typical coiling, suggesting that this gene acts after *whiA*, *whiB*, *whiG*, and *whiJ* (van Wezel et al. 2000). Transcription of *ssgB*, a gene related to *ssgA* and under the control of the stress-responsive sigma factor σ^{H}, is induced at the onset of aerial mycelium formation and is sustained through to the end of sporulation, with maximal promoter activity at the onset of sporulation (Kormanec and Sevcikova 2002*a*). An *ssgB* mutant has a white phenotype similar to an *ssgA* mutant, but an *ssgB* mutant has straight aerial hyphae, an observation suggesting that SsgB is involved in regulating a developmental stage preceding that regulated by SsgA (Keijser et al. 2003). Since colonies of an *ssgB* mutant are larger than those of a wild-type strain, SsgB, like WhiA and WhiB, might be involved in controlling mechanisms of growth cessation, a process that must take place before septum formation can occur (Keijser et al. 2003).

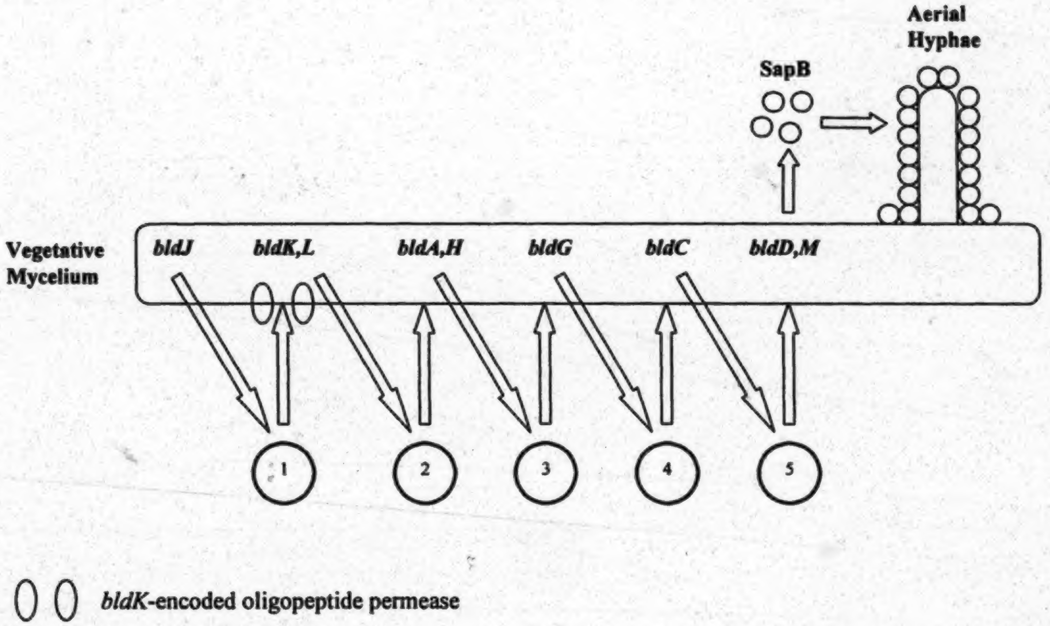
The finding that *ftsZ*, *ssgA*, and *ssgB* are novel *whi* genes supports the idea that the genetic map of *whi* genes is not saturated, and that several other *whi* loci await discovery. Identification of more novel *whi* genes will greatly help unravel the sporulation regulatory network. Many of the *whi* genes encode regulatory proteins, but the targets for these regulators are still largely unknown. Therefore, the discovery of these targets will hopefully lead to greater understanding of the pathways that control sporulation.

1.2.2. Genes controlling aerial mycelium formation

1.2.2.1. The *bld* genes

The *bld* genes are also involved in morphological differentiation and act at an earlier developmental stage than do the *whi* genes, since mutations in the *bld* genes result in a smooth, bald colony appearance due to the lack of fuzzy, white aerial mycelium (Merrick 1976). In addition to this more severe phenotype, *bld* mutants, many of which are deficient in antibiotic production, are also pleiotropically defective in carbon catabolite repression and cell-cell signalling (Pope et al. 1996). The *bld* genes specify 13 distinct loci (*bldA-D*, *F-N*), but *bldF*, *bldI*, and *bldL* have not been extensively studied (Champness 1988; Merrick 1976; Nodwell et al. 1999).

An interesting feature of the *bld* mutants is that they exhibit extracellular complementation. A fringe of aerial mycelium occurs on the proximal edge of substrate mycelial patches of *bld* mutants when they are grown in close proximity to differentiating colonies (Willey et al. 1991). Additionally, the juxtaposition of certain *bld* mutant pairs results in a phenomenon where aerial mycelium formation is restored in one of the mutants (Willey et al. 1993). This observation led to the proposal of an extracellular signalling cascade that involves a donor *bld* mutant giving a signal to a recipient *bld* mutant, allowing the latter to undergo proper differentiation (Willey et al. 1993). This signalling cascade is always unidirectional, forming a hierarchy (*bldJ* < *bldK*, *bldL* < *bldA*, *bldH* < *bldG* < *bldC* < *bldD*, *bldM*) such that mutants can restore aerial mycelium formation to all the mutants to the left, but not to the right. It is further proposed that the *bld* genes are involved in the synthesis and/or release and uptake of extracellular signals, eventually leading to the *bldD*-dependent production of SapB (spore-associated protein), a morphogenetic molecule involved in differentiation (Figure 1.2) (Nodwell et al. 1996; Willey et al. 1993). Some support for this signalling cascade comes from work showing that the *bldK*-encoded oligopeptide permease is responsible for the uptake of a *bldJ*-dependent signal (Nodwell and Losick 1998). Although there is strong biochemical evidence to support the idea that this extracellular signalling cascade is responsible for aerial mycelium formation, there are also several lines of evidence that appear to refute this proposal. For example, *bldB*, *bldI*, and *bldN* do not fit neatly into the extracellular signalling cascade, indicating that this cascade cannot be as simple as proposed (Chater 2001). Furthermore, the *bld* genes are implicated in the production of SapB, based on the observation that all *bld* mutants are deficient in SapB production; however, aerial hyphae formation in *bld* mutants is restored on minimal medium containing mannitol when SapB is not detected, suggesting that alternate pathways are available for proper erection of aerial hyphae (Willey et al. 1991). Probably the most striking evidence against a simple, linear extracellular cascade is the finding that the *bld* genes encode a diverse group of


Aerial
Hyphae
SapB
Vegetative
Mycelium
bldJ
bldK,L
bldA,H
bldG
bldC
bldD,M
1
2
3
4
5
bldK-encoded oligopeptide permease

proteins that do not appear to function in the same way as the BldJ-BldK signal-transporter system.

To date, *bldA* is the best-characterized *bld* gene. *bldA* encodes a leucyl-tRNA required for efficient translation of the TTA codon, which is very rare in the high G + C content of the *Streptomyces* spp. genome (Lawlor et al. 1987). Subsequent studies showed that expression of genes containing TTA codons is reduced or eliminated in a *bldA* mutant (Leskiw et al. 1991). This result, coupled with the observation that a *bldA* deletion mutant is impaired both in aerial mycelium formation and in antibiotic production, led to the hypothesis that the *bldA*-encoded tRNA is required for proper morphological differentiation and secondary metabolism (Leskiw et al. 1991). According to this hypothesis, only genes involved in the above mentioned pathways would contain TTA codons, while genes needed for vegetative growth and primary metabolism would not contain these rare codons. Further studies to characterize the *bldA* transcription start point found that although the *bldA* promoter is always active, mature *bldA*-encoded tRNA is present only when cultures are undergoing morphological differentiation and antibiotic production (Leskiw et al. 1993). In an attempt to define the regulatory connections between *bldA* and other *bld* genes, *bldA* expression was examined in a number of *bld* mutants, and it was shown that *bldA* expression is dramatically decreased in a *bldI* mutant, suggesting that the *bldI*-encoded product may be required for direct or indirect control of *bldA* expression or function (Leskiw and Mah 1995). Recently, a regulatory link between *bldA* and *bldH* was established when it was discovered that the *bldH109* mutant contains a frameshift mutation in the TTA-containing *adpA* gene (A-factor dependent promoter) (Nguyen et al. 2003; Takano et al. 2003). When the TTA codon within the *adpA* gene was changed to an alternate leucine codon, aerial hyphae formation was partially restored in a *bldA* mutant. The *adpA* gene (= *bldH* gene in *S. coelicolor*) encodes AdpA, a transcriptional activator of genes important for differentiation and secondary metabolism in *S. griseus* (Ohnishi et al. 2002). Altogether, these observations strongly support the proposal that the *bldA*-encoded leucyl-tRNA is required for translational regulation of genes controlling morphological differentiation and antibiotic synthesis.

The products of *bldB* and *bldD*, although clearly not homologues, possess similar features in that both proteins contain helix-turn-helix motifs implicated in DNA binding and are capable of dimerization (Eccleston et al. 2002; Elliot et al. 1998; Pope et al. 1998). BldD is the focus of this thesis and will therefore be discussed in greater detail in section 1.6. The *bldB* gene is of particular interest because *bldB* mutants exhibit the most pleiotropic phenotype when compared to other *bld* mutants (Champness 1988; Merrick 1976). In other *bld* mutants, morphological differentiation, and in many cases, antibiotic production, could be restored when grown on poorly used carbon sources. In contrast, when *bldB* mutants are grown under the same conditions, these processes are not restored. BldB is a protein of 99 amino acids shown to exhibit negative autoregulation, since expression of the *bldB* gene is deregulated in a *bldB* mutant (Pope et al. 1998). Expression of *bldB* is developmentally regulated with *bldB* promoter activity being highest at later time points in colony development (Pope et al. 1998). Attempts to show interaction between BldB and the *bldB* promoter in *S. coelicolor* have been unsuccessful (Eccleston et al. 2002), but BldB from the close relative, *Streptomyces lividans*, has been shown to bind to its own promoter, providing evidence that BldB is likely a DNA-binding autoregulatory protein (Mishig-Ochiriin et al. 2003).

The *bldC* locus, which has only been recently characterized, encodes a product that belongs to a previously unknown family of small (58 to 78 amino acids) DNA-binding proteins, whose members contain DNA-binding domains similar to those found in proteins belonging to the MerR family of transcriptional activators (Hunt et al. 2005). *bldC* transcription occurs from a single, constitutively active promoter that is not dependent on any of the known *bld* genes. It is not yet known how BldC exerts its control on morphological differentiation but work by Hunt et al. (2005) has shed some light on the role of this protein in antibiotic synthesis. BldC appears to function in regulating the transcription of the pathway-specific activator gene, *act*II-ORF4, involved in synthesis of the antibiotic actinorhodin. In addition, BldC is needed for maintaining transcription of *redD*, a pathway-specific activator gene involved in the synthesis of the antibiotic undecylprodigiosin, when aerial hyphae are differentiating into spore chains.

The *bldG* gene encodes a putative anti-anti-sigma factor, and is upstream of and co-transcribed with *orf3*, which encodes a putative anti-sigma factor (Bignell et al. 2000). The absence of a nearby sigma factor gene led to the hypothesis that BldG and ORF3 may be involved in the regulation of more than one sigma factor. Two promoters, located upstream of *bldG*, control transcription of *bldG* and *orf3*, giving rise to a polycistronic transcript encoding both BldG and ORF3, and a monocistronic transcript encoding only BldG. BldG is post-translationally modified by phosphorylation, and this phosphorylation is necessary for proper development of *S. coelicolor* colonies (Bignell et al. 2003). To date, it is still not known which kinase is responsible for BldG phosphorylation, whether ORF3 interacts with BldG, and which sigma factor(s) is regulated by BldG.

As mentioned above, *bldK* is a complex locus consisting of a gene cluster containing five ORFs, which are homologues of the subunits of an oligopeptide permease belonging to a family of ATP-binding cassette membrane spanning transporters (Nodwell et al. 1996). Medium conditioned by a *bldK* mutant contains a factor which, when purified, could restore aerial mycelium formation to the *bld261* mutant (now named *bldJ*) (Nodwell and Losick 1998). It is postulated that this factor acts at the first step of the extracellular cascade and that the synthesis of this factor depends on *bldJ*, while the response to this factor depends on a functional *bldK*-encoded oligopeptide permease (Figure 1.2). Very little is known about the *bldJ*-dependent signal; hopefully further characterization of this factor will provide greater understanding of the extracellular signalling cascade. Recently it was suggested that in addition to its proposed role in sensing the *bldJ*-dependent signal, the *bldK*-encoded transporter may also function in sensing and taking up of S-adenosylmethionine, a biological molecule whose accumulation in the cell affects differentiation in *S. coelicolor* (Park et al. 2005).

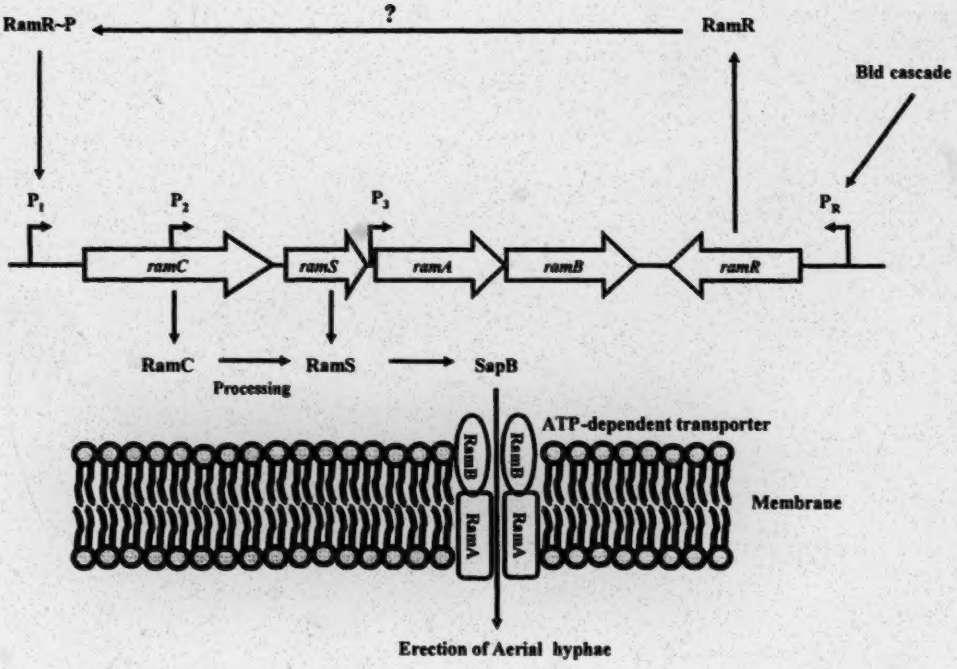
In a screen for white mutants, *whiK* and *whiN* were identified as novel sporulation loci (Ryding et al. 1999). Later, null mutations in these two *whi* loci produced mutants defective in aerial mycelium formation, indicating that these genes are actually novel *bld* genes (Bibb et al. 2000; Molle and Buttner 2000). *bldM*, previously *whiK*, encodes a putative response regulator belonging to the FixJ subfamily of two-component response regulators, with an N-terminal receiver domain containing a conserved acidic phosphorylation pocket (Molle and Buttner 2000). Interestingly, however, BldM could not be phosphorylated *in vitro* and examination of the *S. coelicolor* genome sequence did not reveal a sensor kinase gene adjacent to *bldM*. Similar to BldG, BldM might function in cooperation with multiple sensor kinases; however, further characterization of BldM is needed to determine how this protein controls aerial mycelium formation. *bldN*, previously *whiN*, encodes an extracytoplasmic function (ECF) sigma factor required to initiate aerial mycelium formation, and transcription of this gene is temporally correlated with the onset of aerial mycelium formation (Bibb et al. 2000). *bldN* is involved in a very intricate regulatory network wherein transcription of *bldN* is dependent upon *bldG* and *bldH*, while transcription from one of the two *bldM* promoters depends on σ^{BldN} (Bibb et al. 2000). Additionally, BldD represses *bldN* transcription during vegetative growth (Elliot et al. 2001). The *bldN*-encoded sigma factor has an unusual N-terminal extension of 86 amino acid residues (Bibb and Buttner 2003). The 35 kDa pro-σ^{BldN} is present at early time points during development and is replaced by the 28 kDa mature σ^{BldN} during the formation of aerial mycelium. The protease responsible for cleaving the 35 kDa pro-σ^{BldN} to its mature form has not yet been identified. Since σ^{BldN} is a key player in the initiation of aerial mycelium formation, it would be interesting to identify BldN target genes, although one known BldN target, the *bldMp1* promoter, has already been discovered.

There is still very limited information about how each of the *bld* gene products exerts its control on morphological differentiation. Hopefully, further characterization of each of these proteins might shed light on the regulatory cascade involved in control of morphological differentiation.

1.2.2.2. The *ram* genes

In addition to the *bld* genes, the *ram* genes, which are dispensable for antibiotic production, have been found to affect aerial mycelium formation in *S. coelicolor* and *S. lividans* (Ma and Kendall 1994). These genes are homologues of the *amf* genes (aerial mycelium formation) in *S. griseus* and they are named for the rapid aerial mycelium phenotype that results when they are introduced on a low-copy-number plasmid into *S. lividans* (Ma and Kendall 1994). The *ram* cluster consists of a four gene operon, *ramCSAB,* and a downstream gene, *ramR*, which is transcribed from a convergent promoter (Figure 1.3) (Keijser et al. 2002). *ramCSAB* is under the control of three promoters, with a strong promoter located upstream of *ramC* and two weaker promoters located within *ramC* and upstream of *ramA* (Keijser et al. 2002). Transcription of *ramR* and *ramCSAB* is absent in *bldA*, *bldB*, *bldD*, and *bldH* mutants, but present in a *whiG* mutant (Keijser et al. 2002). This finding, along with the observation that expression of *ramC* is confined to the vegetative hyphae and nonsporulating aerial hyphae (O'Connor et al. 2002), suggests that the *ram* genes provide a developmental link between the *bld* and *whi* genes (Keijser et al. 2002).

ramR encodes a response regulator but is not located next to a sensor kinase gene (Keijser et al. 2002), a situation reminiscent of the *bldM*-encoded response regulator. RamR is a positive regulator of *ramCSAB* transcription and its function is dependent on phosphorylation, but it is not yet known which form of RamR binds to the strong upstream *ramC* promoter to activate transcription or which kinase is responsible for RamR phosphorylation (Keijser et al. 2002; Nguyen et al. 2002; O'Connor et al. 2002). *ramA* and *ramB* encode ATP-binding membrane transport proteins proposed to function


?
RamR~P
RamR
Bld cascade
P_1
P_2
P_3
P_R
ramC
ramS
ramA
ramB
ramR
RamC
Processing
RamS
SapB
ATP-dependent transporter
RamB
RamB
RamA
RamA
Membrane
Erection of Aerial hyphae

in the export of a hydrophobic surface molecule required for coating aerial hyphae (Ma and Kendall 1994). Previous examination of the N-terminal region of RamC led to the proposal that RamC is a membrane-bound serine/threonine kinase needed for proper development (Hudson et al. 2002; Nguyen et al. 2002); however, recent examination of the C-terminal region of RamC identified domains similarly found in proteins involved in the maturation of lantibiotic peptides (Kodani et al. 2004). A closer look at the putative amino acid sequence encoded by *ramS* suggested that this peptide might be a good candidate for the introduction of lantibiotic-like modifications by RamC. Sequence analysis of SapB, a morphogenetic molecule previously resisting sequence identification, revealed that this protein is likely a processed form of the *ramS* gene product. The current hypothesis is that RamC, either functioning alone or in conjunction with other lantibiotic processing enzymes, is responsible for the post-translational modifications needed for conversion of RamS to SapB (Kodani et al. 2004).

1.2.2.3. Genes encoding hydrophobic surface proteins

In addition to the roles played by the *bld* and *ram* gene products, the proper formation and erection of aerial hyphae require the interplay between three classes of hydrophobic proteins. The first group of proteins, known as the Sap proteins, consists of five members, of which SapB is the best-characterized (Willey et al. 1991). Although the genetic determinant for SapB has only been recently elucidated as mentioned above in section 1.2.2.2, SapB has long been considered a key morphogenetic molecule playing a critical role in reducing the surface tension at the medium-air interface, thus allowing aerial structures to erect into the air (Tillotson et al. 1998; Willey et al. 1991). SapB, however, does not appear to contribute to the surface hydrophobicity of aerial hyphae and spores because it could not be detected on the surface of these structures but rather is present predominantly in the surrounding culture medium (Wosten and Willey 2000). More recently, it was shown that the production of the hydrophobic surface layer of *S. coelicolor* aerial hyphae, known as the rodlet layer, requires two homologous proteins, RdlA and RdlB (Claessen et al. 2002). While disruption of *rdlA* and *rdlB* results in loss of the characteristic rodlet layer, formation and differentiation of aerial hyphae is unaffected. Soon after the discovery of the RdlA-B proteins, a third family of eight proteins, called the chaplins (coelicolor hydrophobic aerial proteins) or Chp proteins, was shown to coat aerial structures, and disruptions in the genes coding for these proteins cause a delay in aerial mycelium formation (Claessen et al. 2003; Elliot et al. 2003*a*). However, the fact that multiple (four or greater) gene disruptions are needed to cause a mutant phenotype indicates that these proteins have redundant functions. Like SapB, the chaplins possess surfactant activity, suggesting that they too function in reducing surface tension at the medium-air interface to facilitate the escape of aerial structures into the air. Unexpectedly, however, while exogenous addition of SapB could restore aerial hyphae formation in *bld* mutants (Tillotson et al. 1998), exogenous addition of chaplin preparations was not sufficient for rescuing aerial hyphae formation in the same class of mutants (Claessen et al. 2003). This finding could mean that the chaplins function at a later developmental stage than does SapB. More recently, Claessen et al. (2004) found that the formation of the rodlet layer on the surface of aerial structures requires the expression of both the rodlins and chaplins. The new hypothesis is that the rodlins do not actually form the rodlet layer but rather RdlA-B function to align chaplin fibrils (structures formed from self-assembly of chaplins) into a rodlet layer. This hypothesis is based on the observation that in the absence of RdlA-B, the chaplin fibrils are still present but are distributed randomly on the surface of aerial filaments.

1.3. A connection between stress and development

In *S. coelicolor*, certain heat and salt stimulons are coexpressed with developmental stimulons, suggesting that stress and development are connected in this bacterium (Vohradsky et al. 2000). This could mean that *S. coelicolor* ensures a balance between physiological and environmental conditions before initiating processes involved in morphogenesis (Novotna et al. 2003). Several sigma factors have been implicated in dual regulation of the developmental program and stress regulatory systems.

Transcriptional analysis of *sigH*, encoding an alternative sigma factor σ^H, implicated σ^H in regulating the response to environmental stress (Kormanec et al. 2000). Two observations have led to the proposal that σ^H, in addition to regulating stress responses, is also involved in controlling developmental processes. First, transcription of *sigH* is under the control of two developmentally regulated promoters with *sigHp1* being restricted to vegetative growth, and *sigHp2* being confined to aerial growth and spores (Kelemen et al. 2001). Second, the spatial localization of *sigHp2* activity to aerial hyphae is mediated directly by the developmental transcriptional regulator BldD (Kelemen et al. 2001). σ^H also regulates transcription of *ssgB*, a gene required for sporulation (Kormanec and Sevcikova 2002*a*; Sevcikova and Kormanec 2003), and controls expression of one of the promoters of *gltB*, a gene specifying glutamate synthase needed for production of glutamate, a precursor for the proline osmoprotectant (Kormanec and Sevcikova 2002*b*). These findings provide further evidence for the involvement of σ^H in regulating both developmental processes and stress response systems. A *sigH* disruption mutant, however, has a wild-type phenotype with respect to morphology and salt tolerance, indicating that *S. coelicolor* has other sigma factors possessing redundant functions (Viollier et al. 2003).

σ^R, an ECF sigma factor encoded by *sigR*, is involved in regulating expression of the *p1* promoter of the *trxBA* operon, encoding the thioredoxin system required for the response to oxidative stress (Paget et al. 1998). A mutation in the *rsrA* gene, encoding the antagonist of σ^R, results in loss of sporulation capacity giving a phenotype similar to that of a *whiG* mutant (Kang et al. 1999; Paget et al. 2001). The sporulation-defective phenotype of the *rsrA* mutant could result either from increased levels of free σ^R outcompeting sporulation-specific sigma factors, or from overexpression of σ^R target genes that encode negative regulators of sporulation (Paget et al. 2001).

Unlike the *sigH* and *rsrA* mutants, a *sigB* mutant is severely defective in aerial mycelium formation and has a bald colony phenotype (Cho et al. 2001). *sigB* encodes an alternative sigma factor, σ^B, and one of its promoters is upregulated at the onset of differentiation and is induced by osmotic stress, suggesting that σ^B is required for both proper differentiation and osmoprotection (Cho et al. 2001). Previously it was postulated that σ^B controls these two processes mainly through the regulation of the expression of *catB*, a gene encoding an osmosensitive catalase (Cho et al. 2000, 2001). Recent microarray analysis, however, identified σ^B-dependent induction of more than 280 genes following salt treatment (Lee et al. 2005). In addition to finding genes encoding proteins involved in osmoprotection, genes encoding several other sigma factors and oxidative defence proteins were also identified. It appears then, that the developmental program and stress regulatory systems are more intimately connected than previously believed, and that σ^B functions as a master regulator linking these two important processes together.

Apparently, the developmental switch between vegetative growth and aerial growth requires coordinated control of stress regulatory systems with morphogenesis. An explanation for this phenomenon could be that *S. coelicolor* ensures that environmental conditions are suitable before making the decision to undergo differentiation. This conclusion is plausible because if differentiation is initiated when conditions are not ideal, morphogenesis can prematurely arrest due to insufficient nutrients. Moreover, during differentiation, nutrients for aerial growth arise from the cannibalization of vegetative hyphae. It is therefore likely that colonies undergoing differentiation are more sensitive to harsh environmental conditions than undifferentiated colonies.

Clearly, there are many key players involved in several regulatory pathways directing aerial mycelium formation. There is not yet enough information about the roles played by each of these regulatory elements, making it difficult to understand how all the players fit into the regulatory cascades controlling aerial mycelium formation.

1.4. Regulation of antibiotic biosynthesis

S. coelicolor produces four well-characterized antibiotics. The first antibiotic identified, methylenomycin A, is a cyclopentenone antibiotic encoded by a cluster of genes present on the SCP1 plasmid (Hopwood et al. 1995; Kirby et al. 1975; Wright and Hopwood 1976*a*). The other three *S. coelicolor* antibiotics are chromosomally-determined. Actinorhodin, the second antibiotic discovered, is a diffusible, blue-pigmented polyketide antibiotic synthesized by a group of polyketide synthetases (Hopwood et al. 1995; Wright and Hopwood 1976*b*). Actinorhodin has very low activity against other bacteria, but large amounts of this antibiotic are produced to compensate for this deficiency (Wright and Hopwood 1976*b*). The third characterized antibiotic, undecylprodigiosin, is a red, cell-associated, nonpolar tripyrrole antibiotic that is actually the major component of a group of four prodiginines (Hopwood et al. 1995; Rudd and Hopwood 1980; Tsao et al. 1985). Like actinorhodin, undecylprodigiosin has limited antibiotic activity, probably largely due to the low diffusibility of this antibiotic (Rudd and Hopwood 1980). The final antibiotic, calcium-dependent antibiotic (CDA), is an acidic lipoprotein that acts as a calcium-dependent ionophore to counter Gram-positive bacteria (Hopwood and Wright 1983; Lakey et al. 1983). CDA is synthesized by non-ribosomal peptide synthetases, although other genes are required for synthesis of the unusual amino acids in the structure and for post-assembly modifications of the antibiotic (Chong et al. 1998). The four antibiotics described above belong to distinct chemical classes, so the study of these antibiotics can lead to a general model for the regulation of secondary metabolism in both *S. coelicolor* and other streptomycetes.

As with morphological differentiation, regulation of antibiotic biosynthesis is very complex requiring the action of both pathway-specific and pleiotropic regulators. Pathway-specific regulators control the synthesis of a single antibiotic and are encoded near, usually within, the antibiotic biosynthetic gene cluster that they regulate (Bibb 1996). Pleiotropic regulators are encoded away from the genes that they regulate and are involved in controlling the production of multiple antibiotics or possess a dual function in controlling both morphological differentiation and antibiotic synthesis.

Many of the *bld* mutants (*bldA*, *B*, *D*, *G*, *H*) are pleiotropically defective in aerial mycelium formation and antibiotic production (Champness 1988; Merrick 1976). The mechanism by which *bldA* exerts its control on antibiotic production is the best understood. The *act* gene cluster directing biosynthesis of actinorhodin has two TTA-containing genes including *act*II-ORF4 encoding a pathway-specific activator needed for transcription of the actinorhodin biosynthetic genes, and *act*II-ORF2 encoding a putative export protein for actinorhodin secretion (Fernandez-Moreno et al. 1991). Actinorhodin production, but not export, in a *bldA* mutant is restored when the TTA codon within *act*II-ORF4 is mutagenized to an alternate leucine codon, indicating that the *bldA*-encoded leucyl-tRNA is required for translation of the UUA codon within the *act*II-ORF4 mRNA (Fernandez-Moreno et al. 1991). Although slightly more complicated, *bldA*-mediated translational control is also observed with undecylprodigiosin production (White and Bibb 1997). The *redD* gene, encoding an activator protein for the undecylprodigiosin biosynthetic pathway, is homologous to the *act*II-ORF4 activator gene but it does not contain any TTA codons (Takano et al. 1992). Isolation of *bldA*-derived Pwb mutants (red-pigmented while bald) led to the identification of *redZ*, a gene containing a single TTA codon and encoding a protein typical of response regulators (Guthrie et al. 1998). A regulatory cascade involving *bldA*, *redZ*, and *redD* was then revealed, wherein translation of *redZ* mRNA is dependent on the *bldA*-encoded leucyl-tRNA, and transcription of *redD* is dependent on the activator protein RedZ (Guthrie et al. 1998; White and Bibb 1997).

A-factor (Autoregulatory factor) is a γ-butyrolactone signalling molecule required for streptomycin production and aerial mycelium formation in *S. griseus* (Beppu 1992). ArpA (A-factor receptor protein) is a DNA-binding protein that acts as a negative regulator of streptomycin production and differentiation, and interacts with A-factor leading to derepression of genes involved in the aforementioned processes (Onaka et al. 1995; Onaka and Horinouchi 1997). Two homologues of ArpA found to be involved in secondary metabolism and differentiation were identified in *S. coelicolor* (Onaka et al. 1998). CprA (*S. coelicolor* pigment production regulator) acts as a positive regulator of antibiotic synthesis and morphological differentiation, and CprB acts as a negative regulator of both processes (Onaka et al. 1998).

In contrast to the above described global regulators of secondary metabolite production, many of the other known pleiotropic regulators of antibiotic synthesis are not required for proper morphological differentiation. Several of these global regulators function as two-component regulatory systems. Included are the AfsK-AfsR (A-factor synthesis), AfsQ1-AfsQ2, and AbsA1-AbsA2 (antibiotic synthesis) two-component signal transduction systems. The AfsK-AfsR two-component system is of special interest because it is similar to eukaryotic systems wherein autophosphorylation of the sensor kinase, AfsK, occurs on serine/tyrosine residues (instead of histidine residues), followed by transfer of these phosphates to serine/threonine residues (instead of aspartate residues) of the response regulator, AfsR (Matsumoto et al. 1994). When phosphorylated, AfsR acts as a DNA-binding positive regulator of antibiotic production (Hong et al. 1991). Although playing a role in antibiotic production, the AfsQ1-AfsQ2 system is not obligately required for antibiotic production because disruption of the chromosomal copy of *afsQ1* and *afsQ2* does not cause a detectable change in antibiotic synthesis (Ishizuka et al. 1992). On the other hand, the *absA* and *absB* loci specify the most pleiotropic regulators of secondary metabolite production because mutations in these two loci cause a deficiency in synthesis of all of the four known antibiotics of *S. coelicolor* (Champness et al. 1992).

In summary, several pleiotropic regulators of antibiotic synthesis have been identified, but still very little is actually known of how these regulators exert their control on antibiotic production. Many of these global regulators of secondary metabolism are two-component regulatory systems. Identification of either the signals recognized by these systems or the target genes regulated by these systems should provide great insight into the complex regulatory network controlling secondary metabolite production. Furthermore, since some pathways controlling antibiotic synthesis share regulatory elements with pathways controlling morphological differentiation, greater understanding of one will help provide more information about the other.

1.5. Transcriptional regulators

1.5.1. General features

Transcriptional regulators are DNA-binding proteins that regulate gene expression to modulate growth and development as well as differentiation (reviewed by Pabo and Sauer 1992). Families of DNA-binding proteins are separated based on related structural motifs making up the following classes: the helix-turn-helix proteins, the homeodomains, the zinc finger proteins, the steroid receptors, the leucine zipper proteins, and the helix-loop-helix proteins. In recent years, other families of DNA-binding proteins have been identified and these families have been further classified into smaller groups (reviewed by Luscombe et al. 2000).

Among prokaryotes, the helix-turn-helix (HTH) motif is the most commonly used DNA-binding motif (reviewed by Huffman and Brennan 2002). The classical HTH is made up of a tight, four-residue turn (often with a glycine in the second position) joining two helices arranged at angles of ~120°. The second helix, called the 'recognition helix,' is important for specificity and interacts with the major groove of the DNA. A third helix is needed to stabilize the HTH structure into a compact, globular domain. Many prokaryotic proteins containing the HTH motif bind DNA as a homodimer with each monomer binding one half-site of symmetry-related DNA.

Transcriptional regulators can function as either repressors or activators of gene expression (reviewed by McClure 1985). Repressors bind to operator sites near or overlapping the promoter, and have been found to bind as far upstream as -60 and as far downstream as +12 with respect to the transcription start point. Activators are distinguished from repressors because they bind DNA near or upstream of the -35 region. Analysis of 30 repressors and 94 activators and dual regulators, those that act both as repressors and activators, from *E. coli* showed that in general, repressor proteins contain the HTH motif at the N-terminal end while activators and dual regulators contain the HTH motif at either the N- or C-terminal end (Prag et al. 1997).

1.5.2. Post-translational control of transcriptional regulators

Activity of transcriptional regulators is often controlled by post-translational mechanisms. Probably the three most common post-translational modifications are phosphorylation, proteolytic processing, and protein-protein interactions. Of these three modifications, phosphorylation is the most dominant mechanism to regulate cellular activities.

The regulation of protein activity often involves phosphorylation, which is a covalent modification (reviewed by Cozzone 1998). Phosphorylation is a common theme in the regulation of two-component signal transduction systems. Prototypical prokaryotic two-component regulatory systems involve autophosphorylation of the signal transducer at a conserved histidine residue followed by the transfer of this phosphate to a conserved aspartate residue on a response regulator (reviewed by Raivio and Silhavy 2001). The latter phosphorylation event affects the DNA-binding ability of the response regulator, allowing it to control expression of target genes.

A less common theme in prokaryotes is the phosphorylation of tyrosine residues, although it frequently occurs in eukaryotes (reviewed by Cozzone et al. 2004). Regulation of tyrosine phosphorylation during *Streptomyces* spp. growth has been shown to be important for the shift from primary metabolism and vegetative growth to secondary metabolism and sporulation (Waters et al. 1994). As mentioned above in section 1.4, the AfsK-AfsR two-component signal transduction system required for global regulation of antibiotic synthesis in *S. coelicolor* involves tyrosine phosphorylation of the AfsK protein kinase (Matsumoto et al. 1994). Another multicellular prokaryote, *Myxococcus xanthus*, also possesses two uncharacterized tyrosine-phosphorylated membrane proteins, and the pattern of phosphorylation of these proteins changes during development, suggesting that tyrosine phosphorylation may play a role in the developmental switch between stages of aggregation and sporulation in this organism (Frasch and Dworkin 1996). Protein tyrosine phosphorylation has also been observed in *Caulobacter crescentus*, another bacterium possessing a complex developmental program involving a biphasic life cycle (Wu et al. 1999). *C. crescentus* DivL, although a homologue of prokaryotic histidine protein kinases, requires autophosphorylation on a tyrosine residue for activity. Phospho-DivL then phosphorylates CtrA, a response regulator protein controlling cell-cycle processes, leading to normal growth and cell division.

Proteolysis is an important mechanism to maintain cell homeostasis and optimal metabolic activity (reviewed by Jenal and Hengge-Aronis 2003). The action of proteases can result in either the production of a functional protein or the formation of a non-functional degradation product. In bacteria, proteolysis is often mediated by ATP-dependent proteases, of which the Clp proteases are the best-characterized (reviewed by Porankiewicz et al. 1999). In *E. coli*, there are two types of Clp proteases. The proteolytic subunit, ClpP, associates with either the ClpA or ClpX ATPases. In contrast, the ClpQ proteolytic subunit associates with the ClpY ATPase.

Clp-mediated proteolysis has been shown to play a role in regulating development in a number of bacteria. *S. coelicolor* has two *clpP* genes and disruption of *clpP1* diminishes aerial mycelium formation while overexpression of both *clpP1* and *clpP2* causes accelerated aerial mycelium formation (de Crecy-Lagard et al. 1999). In addition, the bald phenotype of the *clpP1* disruption mutant could not be complemented by the introduction of either *clpP1* or *clpP2* alone, indicating that proper ratios of the ClpP1 and ClpP2 proteins are needed for proper aerial mycelium formation. Taken together, these observations suggest that controlled, timely degradation of certain regulatory proteins is essential for proper morphological differentiation in *S. coelicolor*.

As with *S. coelicolor*, Clp-mediated proteolysis of key regulatory proteins is also important for control of certain cellular processes in *C. crescentus* and *B. subtilis*. In *C. crescentus*, ClpXP-dependent degradation of CtrA, a response regulator protein negatively controlling DNA replication, is required for viability and normal cell division

(Jenal and Fuchs 1998). In *B. subtilis*, a sporulating bacterium, Clp-dependent proteolysis plays a role in the synthesis of the sporulation sigma factors σ^F and σ^E (Msadek et al. 1998). It is thought that in a *clpP* deletion mutant, negative regulators of the sporulation process would accumulate to high levels due to the lack of degradation activity (Msadek et al. 1998).

Interaction of a DNA-binding regulatory protein with another protein or small effector molecule can result in a conformational change of the DNA-binding protein such that DNA-binding activity is either enhanced or abolished (reviewed by Beckett 2001). This mechanism of post-translational control has been observed in *B. subtilis*. SinR (sporulation inhibition) is a 13 kDa dual function regulatory protein that is induced at the end of vegetative growth and functions to switch regulatory activities in response to environmental changes such as nutrient limitation (Gaur et al. 1991). SinR is a DNA-binding protein containing two HTH motifs flanking a potential leucine zipper protein dimerization motif (Gaur et al. 1991). For sporulation to occur, the SinR protein must be inactivated by SinI, functioning as an antagonist of SinR by complexing with SinR to inhibit its DNA-binding capacity (Bai et al. 1993).

Aside from the protein modifications described above, other mechanisms of post-translational control operate in prokaryotic systems, although these have not been well-documented for DNA-binding transcriptional regulators. Recently, combined two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometric analysis of 770 *S. coelicolor* proteins led to the identification of several occurrences of post-translational modifications involving N-acetylation and adenylylation (Hesketh et al. 2002). N-acetylation of the oligopeptide permease BldK-ORFD, and adenylylation of glutamine synthetase I and the GlnK nitrogen regulatory protein were revealed (Hesketh et al. 2002). In addition, protein glycosylation, a common theme in eukaryotes, is becoming an emerging phenomenon in prokaryotes. Glycosylation has been documented in a number of bacteria, and this modification has been shown to be important for virulence in many pathogenic bacteria (reviewed by Benz and Schmidt 2002). Whatever the post-translational modification, this mechanism of regulation is very important for the control of protein activity.

1.6. The transcriptional regulator BldD

Study of the *bldD* locus is of special interest because this gene specifies a transcriptional regulator, BldD, involved in controlling the expression of a number of developmental sigma factor genes (Elliot et al. 2001). Additionally, a *bldD* mutant is pleiotropically defective in aerial mycelium formation and antibiotic production (Merrick 1976), indicating that BldD is involved, directly and/or indirectly, in regulating both of these processes. It is then hoped that the understanding of how BldD exerts its control on target gene expression will lead to further insight into the complex regulatory pathways involved in controlling morphological differentiation and antibiotic synthesis.

BldD is a cytoplasmically-localized protein of 167 amino acid residues with a deduced molecular mass of 18 167 Da (Elliot et al. 1998). This protein contains a DNA-binding domain with an N-terminal HTH motif, and binds to its own promoter at two imperfect inverted repeat sequences between the -10 and -35 regions (Elliot and Leskiw 1999; Elliot et al. 2001). The latter observation, combined with the findings that *bldD* transcript levels are most abundant during vegetative growth and that *bldD* transcription is upregulated in a *bldD* mutant, indicates that BldD is autoregulatory (Elliot et al. 1998).

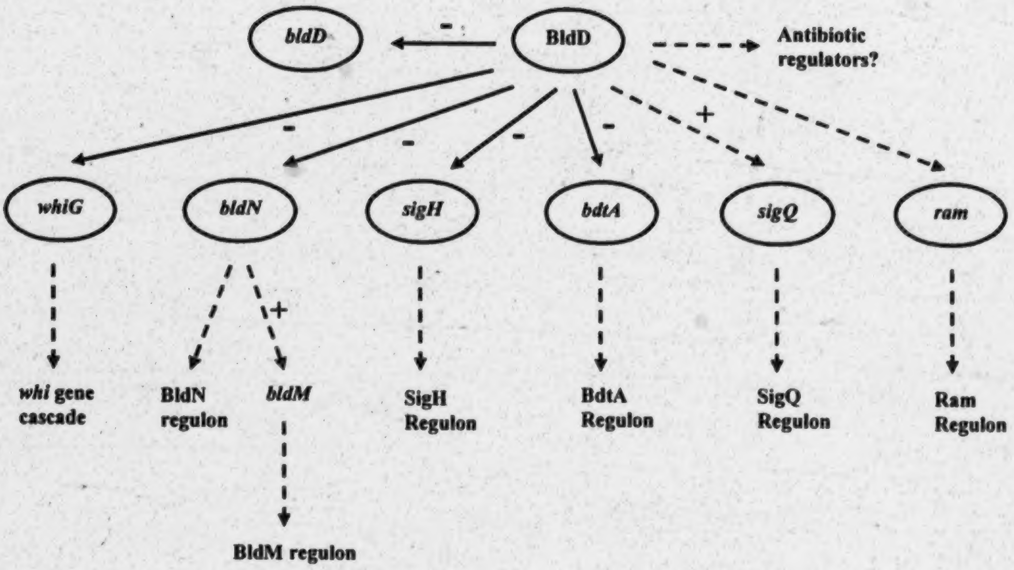
Finally, BldD is dispensable for growth, but required for morphological differentiation and antibiotic synthesis (Elliot et al. 2003*b*).

The first screen for BldD targets identified three genes, including *whiG* and *bldN*, encoding two developmental sigma factors, as well as *bdtA* (BldD target), an uncharacterized gene predicted to encode a transcriptional regulator (Elliot et al. 2001). Transcriptional analysis of *bldN* and *bdtA* showed that these genes are expressed prematurely and at a relatively higher level in a *bldD* mutant compared to the wild-type, indicating that BldD is a transcriptional repressor of these two genes. Similarly, a comparison of the transcriptional pattern for *whiG* in the wild-type and in the *bldD* mutant showed that BldD represses *whiG* expression during vegetative growth. When sporulation begins, however, *whiG* transcript levels decrease in the *bldD* mutant, indicating that BldD might also have an activator effect on this gene, either directly or indirectly. An additional BldD target, *sigH*, encoding a stress-responsive sigma factor, was independently identified by another group (Kelemen et al. 2001). BldD represses expression of *sigH* and mediates spatial and temporal control of this gene from one of the *sigH* promoters (Kelemen et al. 2001).

A *bldD* mutant is defective in both aerial mycelium formation and antibiotic production, indicating that BldD plays a very important role in regulating pathways controlling these two processes, although it is not yet known how BldD exerts its control on antibiotic synthesis. Mentioned above are all the known, characterized BldD targets. These genes are direct BldD targets, as determined by DNA-binding studies, indicating that BldD can bind to the promoter regions of *whiG*, *bldN*, *bdtA*, and *sigH* to affect transcription of these genes (Elliot et al. 2001; Kelemen et al. 2001). As already mentioned, *whiG*, *bldN*, and *sigH* encode sigma factors, and the former two are critical for the initiation of sporulation and aerial mycelium formation, respectively. In controlling expression of these two sigma factor genes, BldD likely indirectly controls expression of a number of other genes essential for proper morphological differentiation (Figure 1.4). In the case of *whiG*, this would mean that transcription of *whiH*, *whiI*, and *sigF* would be affected in a *bldD* mutant since expression of these genes is dependent on σ^{WhiG}. In the case of *bldN*, this would mean that *bldM* transcription would be affected in a *bldD* mutant because σ^{BldN} controls *bldM* expression, while BldD controls *bldN* expression. Taken together, these observations show that BldD manifests its control on morphological differentiation through regulation of expression of several key developmental genes, either directly or indirectly. Understanding the intricate regulatory network surrounding BldD could therefore potentially shed light on the different pathways controlling morphological differentiation.

It is not yet known how BldD controls secondary metabolism because the BldD targets identified to date have not been shown to regulate antibiotic synthesis. It is therefore necessary to do a more exhaustive screen for BldD targets for further insight into how BldD controls antibiotic production.

Earlier work only identified targets that are repressed by BldD. These results were disappointing because based on the observation that a *bldD* mutant is defective in aerial mycelium formation and antibiotic production, it has always been proposed that BldD, in addition to its role as a repressor, must also function as an activator of genes controlling differentiation and secondary metabolism. More recently, microarray analysis of global gene expression in *S. coelicolor* following *bldD* induction revealed that


bldD
-
BldD
Antibiotic regulators?
-
-
-
-
+
whiG
bldN
sigH
bdtA
sigQ
ram
+
whi gene cascade
BldN regulon
bldM
SigH Regulon
BdtA Regulon
SigQ Regulon
Ram Regulon
BldM regulon

expression of *sigQ*, encoding a sigma factor of unknown function, is activated when the BldD protein is overexpressed (C. Galibois, unpublished data). DNA-binding studies have shown that BldD can bind to the promoter region of *sigQ in vitro*, providing further support that this gene is a *bona fide* BldD target (C. Galibois, unpublished data).

The finding that the mutated *bldD* gene contains an A to G substitution at position 62 resulting in a tyrosine to cysteine change, led to the hypothesis that BldD might be modified by phosphorylation at this tyrosine residue (Elliot et al. 2000). However, Western analysis of *S. coelicolor* wild-type cell-free extract using antibodies specifically raised against phosphorylated tyrosine did not reveal a band corresponding to BldD containing a phosphorylated tyrosine residue, indicating that Tyr62 is likely not phosphorylated (Elliot 2000). Recent two-dimensional gel analysis in another lab identified a BldD isoform appearing only at later time points in growth, suggesting that post-translational modification of BldD may be important for proper *Streptomyces* development (A. Hesketh, personal communication, John Innes Centre). This newly-observed BldD isoform is slightly more basic than the unmodified BldD protein and is estimated to be ~1 kDa larger than unmodified BldD, suggesting that the modification is likely not a simple phosphorylation event (80 Da). It is also unlikely that the modification is a simple addition of an acetyl group (42 Da) or an adenyl group (329 Da) (Hesketh et al. 2002). Post-translational control of BldD activity could therefore involve multiple modifications.

1.7. Thesis objectives

The objective of this thesis was to investigate further the role of post-translational modification in controlling BldD activity, either to facilitate relief of BldD repression or to mediate BldD activation of developmental genes. This objective was further broken down into three smaller goals, the first of which was to extend the Hesketh 2D-PAGE study by isolating the putative BldD isoform that accumulates with culture age and characterizing the nature of the BldD modification, if it exists. The second goal was to isolate and characterize any protein(s) that may bind to BldD and modify its structure, and thus regulate its activity. A final aim was to identify a function for the *sigQ*-encoded sigma factor and to validate the previous microarray analysis by confirming that BldD activates the expression of *sigQ*.

CHAPTER 2:

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and cultivation conditions

2.1.1. Bacterial strains

All *E. coli* and *Streptomyces* strains used in this study are listed in Table 2.1.

2.1.2. Plasmids

All *E. coli* and *Streptomyces* plasmids used in this study are listed in Table 2.2.

2.1.3. Growth and maintenance of *E. coli* strains

Culture tubes (18 mm x 150 mm) containing 1-10 mL Luria-Bertani (LB) medium (5 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, pH 7.2) were inoculated either with a single *E. coli* colony growing on solid LB medium containing 1.5% agar (w/v) or with a small amount of *E. coli* glycerol stock (see below). The culture tubes were incubated at 37°C overnight on a rotating wheel. *E. coli* strains harbouring plasmid DNA were cultured in LB medium supplemented with the appropriate antibiotic(s) [100 μg/mL ampicillin (Sigma), 50 μg/mL apramycin (Provel), 25 μg/mL chloramphenicol (Sigma), or 50 μg/mL kanamycin (Sigma)] to maintain plasmids within strains. When required, overnight cultures were diluted 1:50 or 1:100 with fresh LB medium and grown to the desired OD_{600}, either in culture tubes on a rotating wheel or in flasks on a floor shaker. For long term storage of *E. coli* strains, an equal volume of LB overnight culture and 40% glycerol (v/v) were mixed in a 2 mL screw-cap tube (Sarstedt), flash frozen in a dry ice-ethanol bath, and stored at -86°C. *E. coli* cultures growing on solid LB medium were stored at 4°C for short term (1-2 months).

Table 2.1. Bacterial strains used in this study.

Strain	Relevant characteristics(s)	Reference and/or source
E. coli		
DH5α	F$^-$, general cloning host	Hanahan (1983)
ET12567	F$^-$, *dam*, *dcm*, non-methylating host	MacNeil et al. (1992)
JM109	*recA1*, protein overexpression host	Yanisch-Perron et al. (1985)
S. coelicolor		
M600	wild-type, prototroph, SCP1$^-$, SCP2$^-$	John Innes Centre
1169	*bldD* Y62C point mutant	Elliot et al. (1998); Merrick (1976)
ΔbldD1 mutant	*bldD* null mutant in M600 background	Elliot et al. (2003*b*)
ΔbldD FLP mutant (49 or 94)	*bldD* markerless null mutant in M600 background	This study
ΔsigQ mutant	*sigQ* null mutant in M600 background	This study

Table 2.2. ***E. coli*** **and** ***Streptomyces*** **plasmids used in this study.**

Plasmid	Antibiotic marker(s)	Relevant characteristic(s)	Reference and/or source
E. coli plasmids and Streptomyces shuttle plasmids			
pIJ6902	Apramycin, Thiostrepton	P_{tipA} expression vector, multi-copy in *E. coli* and integrates into the ϕC31 *attB* site in *Streptomyces*	Huang et al. (2005)
pAU181	Apramycin	pSET152 derivative (Bierman et al. 1992) with a 1.3 kb *Sph*I-*Xmn*I fragment containing the *bldD* coding region and surrounding flanking upstream and downstream sequence; Multi-copy in *E. coli* and integrates into the ϕC31 *attB* site in *Streptomyces*	Elliot et al. (1998)
pAU243	Ampicillin	pIJ2925 derivative with a 1.3 kb *Bgl*II fragment containing the *bldD* coding region and surrounding flanking upstream and downstream sequence	C. Galibois (unpublished)
pAU244	Apramycin, Thiostrepton	pIJ6902 derivative with a 0.8 kb *Nde*I-*Bgl*II fragment containing the *bldD* coding region with flanking downstream sequence	C. Galibois (unpublished)
pIJ6902::*his-bldD*	Apramycin, Thiostrepton	pIJ6902 derivative with a 0.6 kb *Nde*I-*Bam*HI fragment containing the *bldD* coding region fused to an N-terminal His_6 sequence	This study
pQE9(BldD)$^+$	Ampicillin	pQE9 expression vector (Qiagen) with a 0.5 kb *Hin*dIII-*Bam*HI fragment containing the *bldD* coding region cloned downstream of an N-terminal His_6 tag	Elliot and Leskiw (1999)
pUZ8002	Kanamycin	Helper plasmid encoding transfer functions for mobilization of *oriT*-containing plasmids from *E. coli* to *Streptomyces*	M. Buttner, John Innes Center

Plasmid	Antibiotic marker(s)	Relevant characteristic(s)	Reference and/or source
cosmid SC9C5	Kanamycin	SuperCosI derivative containing *bldD* and flanking sequence from *S. coelicolor*	H. Kieser, John Innes Center
mutagenized cosmid SC9C5	Apramycin, Kanamycin	SC9C5 derivative with the *bldD* coding region replaced by an *aac(3)IV+oriT* cassette (codes for apramycin resistance)	Elliot et al. (2003*b*)
scar-containing cosmid SC9C5	Kanamycin	SC9C5 derivative with the *bldD* coding region replaced by an in-frame 81 bp scar sequence	This study
cosmid 2SCK8	Kanamycin	SuperCosI derivative containing *sigQ* and flanking sequence from *S. coelicolor*	H. Kieser, John Innes Center
Streptomyces plasmids			
pJ4123	Kanamycin, Thiostrepton	High-copy P_{tipA} expression vector	Takano et al. (1995)
pSH19	Thiostrepton	Hyper-inducible expression plasmid using the P_{nitA}-NitR system	Herai et al. (2004)
pSH19::*bldD*	Thiostrepton	pSH19 derivative with a 0.8 kb fragment containing the *bldD* coding region and flanking downstream sequence	This study

2.1.4. Growth and maintenance of *Streptomyces* strains

Surface cultures were typically grown at 30°C on solid sucrose yeast extract (R2YE) medium containing 2.2% agar (w/v) (Kieser et al. 2000). When plasmid selection was necessary, the medium was supplemented with the appropriate antibiotic(s) [50 μg/mL apramycin, 200 μg/mL kanamycin, 25 μg/mL nalidixic acid (Sigma), or 30 μg/mL thiostrepton (Sigma)]. Solid medium cultures were stored at 4°C for 3-6 months. For longer term storage, *Streptomyces* strains were stored as frozen glycerol stocks after harvesting mycelial fragments or spores as detailed below in section 2.1.5.

Small scale (5-10 mL) liquid cultures were grown at 30°C on a floor shaker (240 rpm) in Universals (25 mL glass bottles; Astell Scientific) containing a 1 inch spring coil to disperse the mycelia. Larger scale liquid cultures (25-150 mL) were grown in flasks with a spring coil wrapped around the inside bottom of the container. Cultures were typically grown in R2YE medium (Kieser et al. 2000) or less typically in a 3:2 mixture of yeast extract malt extract:trypticase soy broth (YEME:TSB) (Kieser et al. 2000). Cultures were inoculated either with thawed *Streptomyces* glycerol stocks (50-100 μL spores or 100-500 μL mycelia) or with a loopful of *Streptomyces* biomass growing on solid medium. When necessary, starter seed cultures were grown in yeast extract tryptone (2X YT) medium (Kieser et al. 2000) for 16-24 hours, the mycelia collected, and used to inoculate R2YE or YEME:TSB cultures.

2.1.5. Preparation of *Streptomyces* mycelial and spore stocks

Streptomyces strains were grown on solid R2YE medium overlaid with a cellophane disc (Cannings Packaging Ltd., UK). Mutant bald strains were incubated at 30°C for 1.5-2 days and the biomass was scraped into a 15 mL Duall® tissue grinder

(Kontes Glass Company). The biomass was homogenized in 2-10 mL mQH_2O and the homogenate transferred to a Universal. The tissue grinder was rinsed with additional mQH_2O and this water was pooled with the homogenate in the Universal. The homogenate was centrifuged for 10 minutes at 3000 rpm and 4°C in an International centrifuge model PR-J (International Equipment Company) to collect the mycelia. After washing the mycelia with 10-15 mL 10.3% sucrose (w/v), the mycelia was resuspended in an equal volume of 40% glycerol (v/v) to give a final concentration of 20% glycerol (w/v). Small aliquots (1-4 mL) of the mycelial glycerol stock were transferred to Bijou bottles (7 mL glass bottles; Astell Scientific) and stored at -20°C.

Sporulating *Streptomyces* strains were grown for 4-5 days to allow for full sporulation and the biomass was scraped into a Universal containing 5-10 mL mQH_2O. The biomass suspension was agitated briefly on a vortex mixer and placed in a Bransonic 200 sonicating water bath (Branson) for 10-15 minutes. The suspension was placed on ice to allow settling of the biomass, after which time, the liquid was filtered through nonabsorbent cotton. The filtrate containing the spores was collected in a Universal and centrifuged for 10 minutes at 3000 rpm and 4°C to pellet the spores. The supernatant was discarded and the pellet was resuspended in the remaining liquid before addition of an equal volume of 40% glycerol (v/v) to give a final concentration of 20% glycerol (w/v). Small aliquots (100-500 µL) of the spore suspension were transferred to Bijou bottles and stored at -20°C.

2.2. DNA methods

2.2.1. DNA isolation from bacteria

2.2.1.1. Isolation of plasmid DNA from *E. coli*

Isolation of plasmid DNA from *E. coli* followed the alkaline lysis method outlined by Sambrook et al. (1989).

2.2.1.2. Isolation of plasmid DNA from *Streptomyces*

Preparation of plasmid DNA from *Streptomyces* strains involved the use of the alkaline lysis method detailed by Sambrook et al. (1989) with a few modifications. *Streptomyces* cultures (5-100 mL) were grown to high cell density and the cells were recovered by centrifugation then resuspended in 0.1-5 mL solution I containing 2 mg/mL lysozyme. The resulting cell suspension was incubated in a 37°C water bath for 30 minutes. The remainder of the procedure was performed as detailed by Sambrook et al. (1989) with adjustments in volumes of solution II and III to accommodate changes in the volume of solution I used.

2.2.1.3. Isolation of chromosomal DNA from *Streptomyces*

Total *Streptomyces* DNA was isolated using procedure 3 as outlined by Hopwood et al. (1985) with adjustments in reagent volumes to accommodate a smaller scale isolation procedure. *Streptomyces* strains were grown to high cell density (24-72 hours) in 5 mL R2YE medium. The mycelium was recovered, washed with 2 mL 10.3% sucrose (w/v), and resuspended in 1 mL lysozyme solution. The cell suspension was incubated in a 37°C water bath for 10 minutes then mixed with 1.33 mL 2X Kirby's mix on a vortex mixer for 1 minute. To the mixture, 2.67 mL Tris-buffered phenol:chloroform:isoamyl alcohol (25:24:1) was added and the contents were mixed on a vortex mixer for another

15-30 seconds. This mixture was then centrifuged for 10 minutes at 3000 rpm and 4°C in an International centrifuge model PR-J. The liquid above the interface was extracted once or twice more with 3 mL Tris-buffered phenol:chloroform:isoamyl alcohol until there was virtually no interface between the phases and the aqueous layer appeared mostly clear. The aqueous top layer was then precipitated with 1 volume isopropanol and 1/10 volume 3 M NaOAc (pH 5.2) at room temperature for 10 minutes. The DNA was recovered by centrifuging for 10 minutes at 3000 rpm and 4°C. The pellet was washed with 5 mL 95% ethanol, redissolved in 500 μL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 40 μg/mL DNase-free RNase A (Sigma), and incubated in a 37°C water bath for 30 minutes. The RNA-digested genomic DNA preparation was transferred to a 1.5 mL polypropylene microcentrifuge tube (Fisher Scientific), extracted once with 1 volume Tris-buffered phenol:chloroform:isoamyl alcohol then once with 1 volume chloroform:isoamyl alcohol (24:1). The aqueous top layer was then precipitated with 1 volume isopropanol and 1/10 volume 3 M NaOAc (pH 5.2) as described above. The precipitated DNA was washed first with 100 μL 95% ethanol then with 100 μL 70% ethanol and finally air dried. The genomic DNA preparation was redissolved in 100 μL TE buffer and stored at 4°C. DNA was quantified by determining the absorbance at 260 nm using a UV spectrophotometer (Ultraspec 3000; Pharmacia Biotech).

2.2.2. DNA purification following gel electrophoresis

2.2.2.1. DNA electrophoresis

DNA fragments smaller than 800 bp were separated on 5-8% polyacrylamide gels (29:1 acrylamide:N,N'-methylene bisacrylamide) using the Mini-PROTEAN® 3 electrophoresis system (Bio-Rad). For visual assessment of DNA progression, each sample was mixed with 1/5-1/10 volume DNA loading dye [60% sucrose (w/v), 0.025% bromophenol blue (w/v)]. The gels, which contained a 1X concentration of TBE buffer (89 mM Tris-Cl, 89 mM boric acid, 2 mM EDTA, pH 8.0), were electrophoresed in 1X TBE buffer at 200 V for 20-25 minutes until the dye front was approximately 1 cm from the bottom of the gel. For size determination of fragments, Marker V or Marker XIV (Roche) was used. DNA fragments larger than 800 bp were separated on 0.7-1.2% TBE agarose gels (also containing a 1X concentration of TBE buffer) in 1X TBE buffer at 97-107 V until the dye front was 0.5-1.5 cm from the bottom of the gel. To determine fragment sizes, λ DNA digested with *Pst*I enzyme was used as a molecular weight marker. DNA bands were visualized following staining with ethidium bromide and exposure to UV illumination.

2.2.2.2. Purification of DNA from polyacrylamide gels

Small DNA fragments (60-800 bp) were purified using a modified procedure of the crush and soak purification technique developed by Maxam and Gilbert (1977) and described by Sambrook et al. (1989). DNA fragments were separated on a 5-8% polyacrylamide gel as described in section 2.2.2.1, stained with ethidium bromide, visualized by UV illumination, and the band of interest was excised from the gel. The gel slice was placed into a 0.6 mL tube (Axygen Scientific) containing a needle point hole at the bottom of the tube. The 0.6 mL tube was then placed into a 1.5 mL polypropylene microcentrifuge tube and the gel slice was crushed and transferred to the larger tube by centrifuging the 2 tubes in a microcentrifuge at maximum speed for 1 minute. The crushed acrylamide was then incubated in 2 volumes crush and soak elution buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 8.0) at 37°C overnight on a rotating wheel.

Following incubation, the acrylamide was pelleted by centrifugation and the supernatant was collected in a fresh 1.5 mL polypropylene microcentrifuge tube. The acrylamide was resuspended in another 1 volume crush and soak elution buffer by vortex mixing. Once again the acrylamide was pelleted, the supernatant was collected, and pooled with the supernatant from the first spin. DNA in the supernatant was precipitated on ice for 30 minutes with 2 volumes 95% ethanol and 20 μg glycogen (Roche). The DNA was recovered by centrifugation, redissolved in 200 μL TE buffer, and precipitated once more on ice for 30 minutes with 2 volumes 95% ethanol, 1/10 volume 3 M NaOAc (pH 5.2), and 20 μg glycogen. The precipitated DNA was washed with 70% ethanol, air dried, and redissolved in an appropriate volume of mQH_2O. The DNA preparation was used immediately or stored at -20°C.

2.2.2.3. Purification of DNA from agarose gels

DNA fragments larger than 800 bp and smaller than 2 kb were typically purified using the QIAquick® gel extraction kit purchased from Qiagen. First, DNA fragments were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV illumination as described in section 2.2.2.1. The fragment of interest was excised from the gel, placed into a 1.5 mL polypropylene microcentrifuge tube, and DNA was recovered from the gel slice using the QIAquick® gel extraction kit according to the manufacturer's directions.

DNA fragments larger than 2 kb were typically purified using the trough purification method described by Zhen and Swank (1993). DNA fragments were electrophoresed in a TAE-buffered agarose gel in 1X TAE buffer (40 mM Tris-Cl, 40 mM acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide in the dark at 97-107 V.

Electrophoresis was continued until the dye front was 1-2 cm from the bottom of the gel, at which time, the gel was removed from the gel tank and exposed to UV illumination to visualize the position of the DNA fragment to be purified. A narrow trough was cut immediately below the DNA fragment of interest and the gel was returned to the gel tank. Buffer in the gel tank was removed to drop the buffer level slightly below the top of the gel before filling the trough with PEG trough buffer [15% PEG-8000 (w/v), 0.5 µg/mL ethidium bromide, 1X TAE buffer] and the voltage was increased to 137 V to recover the DNA in the trough. DNA migration into the trough was monitored using a hand-held UV illuminator (Mineralight® Lamp UVSL-25; Ultra Violet Products Inc.). Once the DNA had migrated into the trough buffer, the buffer was transferred into a 1.5 mL polypropylene microcentrifuge tube and extracted first with 1 volume Tris-buffered phenol:chloroform:isoamyl alcohol (25:24:1) then with 1 volume chloroform:isoamyl alcohol (24:1). The aqueous layer was then precipitated on ice for 30 minutes with 2 volumes 95% ethanol and 1/10 volume 3 M NaOAc (pH 5.2). The DNA was recovered, washed with 70% ethanol, redissolved in an appropriate volume of mQH_2O, and finally stored at -20°C or used immediately.

2.2.3. Polymerase chain reaction (PCR)

All oligonucleotide primers used for DNA amplification in this study are listed in Table 2.3. For cloning purposes, PCR reactions used the Expand™ High Fidelity polymerase kit (Roche), which contained a polymerase possessing proofreading ability. The reactions were done in 0.2 mL thin wall PCR tubes (Axygen Scientific) typically with a 50 µL reaction volume containing 0.2 mM dNTPs, 0.4 µM of each primer, 1X Expand™ High Fidelity buffer 2 containing 1.5 mM $MgCl_2$, 0.61 units Expand™ High

Table 2.3. Oligonucleotide primers used in this study.

Primer	Sequence (5'-3')	Region of Homology	Purpose
BKL54	ccgccttcgccaccggt	Internal region of 16S rRNA	Probe for 16S rRNA
BKL104	gcaaaacctcatacagaaaattc	Flanks polylinker of pIJ6902 (downstream of *Bgl*II restriction site)	Sequencing pIJ6902 insert
BKL105	cacgcggaacgtccgggcttgcac	Flanks polylinker of pIJ6902 (upstream of *Nde*I restriction site)	Sequencing pIJ6902 insert
CGA40	gggcgcgagatgaacacg	-150 to -132 from *sigQ* translation start point	Amplifying 170 bp fragment spanning *sigQ* promoter region; ChIP PCR
CGA41	ttcgcgtcaaccaccgtc	+3 to +20 from *sigQ* translation start point	Amplifying 170 bp fragment spanning *sigQ* promoter region; ChIP PCR, DNA affinity chromatography
CGA42	cggaggcgggcgcggacg	+68 to +85 from *prsI* translation start point	Amplifying 162 bp fragment spanning *prsI* promoter and upstream coding regions; ChIP PCR
CGA43	ccctgaaccggctgtgtacg	-77 to -57 from *prsI* translation start point	Amplifying 162 bp fragment spanning *prsI* promoter and upstream coding regions; ChIP PCR
CGA47	cggtgggaaggcgtagtg	-65 to -48 from *arsI* translation start point	Amplifying 69 bp fragment spanning *arsI* promoter region; ChIP PCR
CGA48	ccatgccgtttctctctc	-14 to +4 from *arsI* translation start point	Amplifying 69 bp fragment spanning *arsI* promoter region; ChIP PCR
CGA52	gaaccccggtgtgcgtgc	-85 to -68 from *sigQ* translation start point	Amplifying 83 bp fragment spanning *sigQ* promoter region; DNA affinity chromatography
hrdBF	aagctgaccagattccggc	+125 to +143 from *hrdB* translation start point	Amplifying 101 bp fragment spanning *hrdB* upstream coding region; RT-PCR
hrdBR	ctctgcggcactgaccatc	+207 to +225 from *hrdB* translation start point	Amplifying 101 bp fragment spanning *hrdB* upstream coding region; RT-PCR

Primer	Sequence (5'-3')	Region of Homology	Purpose
KC3	**cgcggtatac**tcgggacggggctgctgac*	-152 to -134 from *afsR* translation start point	Amplifying 233 bp fragment spanning *afsR* promoter and upstream coding regions; ChIP PCR
KC4	gcgcccgattcctcctcttc	+52 to +71 from *afsR* translation start point	Amplifying 233 bp fragment spanning *afsR* promoter and upstream coding regions; ChIP PCR
KC9	**gcgcagatct**agggcaaggtcgtggaggtg	+58 to +77 from SCF55.32 translation start point	Amplifying 277 bp fragment spanning SCF55.32 promoter and upstream coding regions; ChIP PCR
KC10	cgctccaggtcgtcggtcac	-190 to -170 from SCF55.32 translation start point	Amplifying 277 bp fragment spanning SCF55.32 promoter and upstream coding regions; ChIP PCR
KGI6	ccgggcttgcaggcgagtgag	Internal sequence of *aac(3)IV+oriT* cassette	PCR confirmation for FLP excision of *aac(3)IV+oriT* cassette
LB4	**gcgaattccatatg**gcagcagccatcatcatcatcatcacagcagcggcctggtgccgcgcggcagccaCatgtccagcgaatacgccaaac**	His$_6$ sequence with thrombin cleavage site from pIJ4123 followed by the first 22 nt of the *bldD* coding region	Amplifying 584 bp fragment containing *bldD* coding region fused to N-terminal His$_6$ tag with thrombin cleavage site; Cloning His$_6$-BldD expression vector
LB5	cgtcagcacaccgcgtac	-18 to -1 from *sigQ* translation start point	Amplifying 125 bp fragment spanning *sigQ* upstream coding region; RT-PCR
LB6	cggcgctcctggacgtag	+90 to +107 from *sigQ* translation start point	Amplifying 125 bp fragment spanning *sigQ* upstream coding region; RT-PCR
LBU1	gggcgcgagatgaacacg	Complementary to CGA40 sequence containing a 5' biotin unit	Amplifying 83 bp or 170 bp fragment spanning *sigQ* promoter region; DNA affinity chromatography
LBU2	ggaagagtcggtgcgga	Complementary to MAE1 sequence containing a 5' biotin unit	Amplifying 106 bp fragment spanning *bldD* promoter region; DNA affinity chromatography

Primer	Sequence (5'-3')	Region of Homology	Purpose
MAE1	ggaagagtcggtgcgga	-125 to -109 from *bldD* translation start point	PCR confirmation for FLP excision of *aac(3)IV+oriT* cassette
MAE4	**tctaga**gcggcaggctgtgttgtc	-43 to -26 from *bldD* translation start point	Amplifying 106 bp fragment spanning *bldD* promoter region; DNA affinity chromatography
MAE11	cggtagcaggctcacag	-231 to -215 from *bldD* translation start point	Amplifying 258 bp fragment spanning *bldD* promoter region; ChIP PCR
MAE12	gagctgtttggcgtattcg	+9 to +27 from *bldD* translation start point	Amplifying 258 bp fragment spanning *bldD* promoter region; ChIP PCR
MAE14	**ggtggatcc**tcagagctcgtcgtggg	Overlaps *bldD* stop codon	Amplifying 584 bp fragment spanning *bldD* coding region; Cloning His$_6$-BldD expression vector
MAE44	ggatttctggcagttgagg	-245 to -223 from *whiG* translation start point	Amplifying 354 bp fragment spanning *whiG* promoter and upstream coding regions; ChIP PCR
MAE45	tacgaccgccacagctcg	+93 to +112 from *whiG* translation start point	Amplifying 354 bp fragment spanning *whiG* promoter and upstream coding regions; ChIP PCR
MAE68	cgtagccaggcccgagg	-236 to -220 from *bldN* translation start point	Amplifying 208 bp fragment spanning *bldN* promoter region; ChIP PCR
MAE71	gtcaatcgggcacagaagc	-427 to -409 from *bldN* translation start point	Amplifying 208 bp fragment spanning *bldN* promoter region; ChIP PCR

* Restriction endonuclease recognition sites are indicated by underlined text while nonhomologous sequences are shown as bolded text

** Mutagenized nucleotide is indicated by an upper case letter

Other notes:

Primers designated CGA, KC, and KGI were provided by C. Galibois, K. Colvin, and K. Gislason, respectively, of the Leskiw lab.
Primers designated BKL and MAE belong to the collection of Leskiw lab primers.
Primers designated LB or LBU were designed by the author during the course of this study.

Fidelity polymerase, and 1 μg denatured chromosomal DNA or ~0.05 μg plasmid DNA as template. Denaturation of chromosomal DNA was achieved by treating 1-10 μg chromosomal DNA with 40 μmoles NaOH and 0.04 μmoles EDTA (pH 8.0) in a 100 μL reaction volume for 10 minutes at room temperature. The DNA was then precipitated on ice for 30 minutes with 2 volumes 95% ethanol and 1/10 volume 3 M NaOAc (pH 5.2), recovered by centrifugation, and redissolved in water to give a final concentration of 0.2 μg/μL. When necessary, the PCR reactions were supplemented with 2-6% DMSO (v/v) and/or 0.5-2 M betaine and/or 0.5-2 mM $MgCl_2$. In cases when PCR was used for the purpose of screening cloned DNA, the reaction volume was scaled down to 20 μL, adjusting all components for the reduced volume and using 0.5-1 unit in-house Taq polymerase (provided by M. Pickard, Department of Biological Sciences, University of Alberta) in place of the Expand™ High Fidelity polymerase. The reactions were typically subjected to 5 minutes denaturation at 95°C followed by 30 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 52-62°C (2-10°C below primer T_m values), and extension for 1 minute at 72°C, then subjected to a final 10 minutes at 72°C. Thermocycling was done in a Biometra® T personal PCR machine (Montreal Biotech Inc.). The reactions were analyzed by gel electrophoresis immediately or stored at -20°C for later analysis.

2.2.4. Automated DNA sequencing

DNA sequencing was performed using the DYEnamic™ ET kit (Applied Biosystems). Reactions were done in 0.2 mL thin wall PCR tubes with a total volume of 20 μL containing 5 pmoles primer, 5% DMSO (v/v), 8 μL ET mix, and the appropriate amount of template (100-500 ng plasmid DNA or 50-200 ng PCR product). When the primer T_m was below 60°C, the reactions were subjected to 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 1 minute. When the primer T_m was above 60°C, the cycling conditions consisted of 30 cycles of 96°C for 30 seconds and 60°C for 2 minutes. Following thermocycling, sequencing reactions were mixed with 2 µL NaOAc/EDTA (provided with the ET kit) and 80 µL 95% ethanol in a 1.5 mL polypropylene microcentrifuge tube then placed on ice for 15 minutes. The tube was centrifuged for 15 minutes at maximum speed to collect the DNA. The DNA pellet was washed with 500 µL 70% ethanol, air dried briefly, and brought to the Molecular Biology Service Unit (MBSU) (Biological Sciences Building, University of Alberta) for analysis on a sequencing gel.

2.2.5. DNA digestion, ligation, and transformation

2.2.5.1. Restriction enzyme digestion

Restriction digestion of DNA was carried out using reaction conditions recommended by the manufacturers (New England Biolabs, Promega, Roche).

2.2.5.2. DNA ligation

DNA ligations were performed using T4 DNA ligase purchased from Roche. For sticky end ligations, insert DNA and vector DNA were mixed using a ratio of 3:1 in a 10 µL reaction mix containing 1X T4 DNA ligation buffer, 1mM ATP, and 1 unit T4 DNA ligase. The reaction mix was incubated in a 15°C water bath overnight. When necessary, cohesive overhangs were removed by treating the DNA with 1 unit Klenow enzyme (Roche) in a 30 µL reaction mixture containing approximately 1 µg DNA in 1X filling buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 50 µg/mL BSA) before addition of 2 µL 0.125 mM dNTPs. Following addition of the dNTPs and incubation in a

37°C water bath for 15 minutes, the reaction mix was extracted once with Tris-buffered phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1) then precipitated on ice for 30 minutes with 2 volumes 95% ethanol, 1/10 volume 3 M NaOAc (pH 5.2), and 20 μg glycogen before redissolving in mQH_2O. For blunt-sticky end ligations, insert DNA and vector DNA were mixed in a ratio of 10:1 in the same ligation reaction mix as stated above. The reaction mix was then incubated at room temperature for 3-4 hours followed by incubation in a 15°C water bath overnight. To minimize vector recircularization, vector DNA was treated with shrimp alkaline phosphatase (Roche) according to the manufacturer's directions. Ligation reactions were used immediately in DNA transformation reactions or stored at 4°C or -20°C for later use.

2.2.5.3. Preparation of *E. coli* competent cells

For preparation of chemically competent cells, 5 mL LB medium [supplemented with the appropriate antibiotic(s) when required] was inoculated with a single colony of a desired *E. coli* strain and incubated at 37°C overnight on a rotating wheel. The overnight culture was then diluted 1:100 in a total volume of 20 mL LB medium and grown at 37°C on a floor shaker at 220-240 rpm for 3-4 hours until the culture reached an OD_{600} of ~0.6. The culture was transferred to a Universal and centrifuged for 5 minutes at 3000 rpm and 4°C in an International centrifuge model PR-J. The medium was decanted and the cells were washed once with 5 mL cold 100 mM $MgCl_2$ and once with 5 mL cold 100 mM $CaCl_2$. The cells were then resuspended in a 500 μL mixture of 100 mM $CaCl_2$ and 20% glycerol (v/v). The cell suspension was divided into 50 μL aliquots in 1.5 mL polypropylene microcentrifuge tubes, frozen quickly in a dry ice-ethanol bath, and stored at -86°C.

For preparation of electrocompetent cells, a 20 mL LB culture of a desired *E. coli* strain was prepared as described above. The culture was transferred to a Universal and centrifuged for 5 minutes at 3000 rpm and 4°C. The medium was decanted and the cells were washed twice with 10 mL cold 10% glycerol (v/v). The cells were resuspended in 500 µL cold 10% glycerol (v/v) and small aliquots of the cell suspension were stored as described above for chemically competent cells.

2.2.5.4. Transformation of *E. coli*

For introduction of ligation mixtures into *E. coli*, 5 µL of a ligation reaction was mixed with 50 µL freshly thawed chemically competent *E. coli* cells in a 1.5 mL polypropylene microcentrifuge tube. The cell/DNA mixture was incubated on ice for 30 minutes, then heated in a 42°C water bath for 45 seconds and placed on ice for 2 minutes before adding 950 µL room temperature LB medium. The cell mixture was incubated for 1 hour at 37°C on a rotating wheel. Different volumes and dilutions of the cell mixture were spread onto solid LB medium containing the appropriate antibiotic for selection of *E. coli* colonies harbouring recombinant plasmid DNA. For plasmids containing the *lacZ'* gene within the polylinker region, which allowed for blue-white selection, cell mixtures were plated on solid LB medium containing the appropriate antibiotic as well as 40 µg/mL X-Gal and 100 µM IPTG. Plates were incubated at 37°C overnight and *E. coli* colonies were selected for confirmation of presence of recombinant plasmids by first growing all suspected positive clones in liquid medium, isolating plasmid DNA, and performing restriction digestions and/or PCR analysis, followed by DNA sequencing, where appropriate, to ensure sequence integrity.

For introduction of plasmid or cosmid DNA into *E. coli*, 1-2 μL of the DNA preparation was mixed with a 50 μL aliquot of freshly thawed electrocompetent *E. coli* cells. The mixture was transferred into a cold electroporation cuvette (2 mm gap; Molecular BioProducts) and subjected to a brief electrical pulse using a Micropulser™ (Bio-Rad) on program Ec2. The mixture was then combined with 950 μL cold LB medium in a 1.5 mL polypropylene microcentrifuge tube and incubated for 1 hour at 37°C on a rotating wheel. The cell mixture was spread on solid LB medium and incubated as described above.

2.2.5.5. Preparation of *Streptomyces* protoplasts

Streptomyces protoplasts were prepared according to Hopwood et al. (1985).

2.2.5.6. Transformation of *Streptomyces*

Transformation of *Streptomyces* followed the method of protoplast transformation ("original procedure") outlined by Hopwood et al. (1985) with a few modifications. A 1 mL aliquot of frozen *Streptomyces* protoplasts was quickly thawed under cold tap water and then transferred to a 15 mL round bottom polystyrene culture tube (Corning). The culture tube was centrifuged in an International clinical centrifuge model CL (International Equipment Company) for 7 minutes at 3000 rpm to collect the protoplasts. Following introduction of DNA, protoplasts were spread on 1 or 2 plates containing exactly 25 mL solid R2YE medium and grown at 30°C for 16-20 hours without antibiotic selection. The plate(s) was then flooded with 1 mL mQH_2O containing the appropriate amount and type of antibiotic. The antibiotic mixture was allowed to soak into the R2YE agar and incubation at 30°C continued until colonies formed. Transformants were patched by toothpick to solid R2YE medium containing the appropriate antibiotic selection. Transformants harbouring recombinant plasmid DNA were confirmed by restriction digestions and/or PCR analysis following plasmid isolation. Where appropriate, DNA sequencing was performed to ensure sequence integrity.

2.2.6. Introduction of DNA into *Streptomyces* by conjugation

Introduction of DNA into *Streptomyces* by conjugation was performed according to the method described by Kieser et al. (2000) with some modifications. Plasmid DNA to be introduced into *Streptomyces* was first transformed into *E. coli* ET12567/pUZ8002, a non-methylating *E. coli* strain containing a helper plasmid for mobilization of *oriT*-containing plasmids from *E. coli* to *Streptomyces*. The resulting strain harbouring the plasmid of interest was grown and the cells were prepared for conjugation. In a 15 mL round bottom polystyrene culture tube, 500 μL *E. coli* cells was mixed either with 50 μL pre-germinated spores (heat treated for 10 minutes in a 50°C water bath) or with 500 μL thawed mycelial fragments. The cell mixture was collected by centrifugation, the supernatant was decanted, and the cells were resuspended in the remaining liquid. One hundred microlitre volumes of the undiluted cells and of a 10^{-1} dilution were spread onto plates containing exactly 25 mL mannitol-soy (MS) agar + 10 mM $MgCl_2$ (Kieser et al. 2000). The plates were incubated for 16-20 hours without antibiotic selection and then flooded with 1 mL mQH_2O containing 0.5 mg nalidixic acid (inhibits *E. coli* growth) and 1.25 mg apramycin (selects for pIJ6902 and its derivatives). The antibiotic mixture was allowed to soak into the MS plates and incubation at 30°C continued until colonies formed. Colonies were repatched at least twice to solid medium (R2YE, DNA, or MS;

Kieser et al. 2000) containing 25 μg/mL nalidixic acid and 50 μg/mL apramycin. Integration of plasmid DNA into the chromosomes of positive transconjugants was confirmed by PCR analysis on isolated chromosomal DNA.

2.2.7. DNA hybridization

2.2.7.1. *E. coli* colony blot hybridization

For large scale screening of *E. coli* colonies containing recombinant plasmids, colony blot hybridization was performed. An ethanol-proof pen was used to draw a grid onto circular nylon hybridization transfer membranes (Hybond™-N; Amersham Biosciences) to accommodate 50 *E. coli* streaks. The membrane was overlaid onto solid LB medium containing the appropriate antibiotic for plasmid selection. An acetate grid was taped onto the back of the agar side of another LB plate containing the appropriate antibiotic. Sterile toothpicks were used to replica patch *E. coli* transformants first to the LB plate containing the nylon membrane and second to the LB plate with the taped acetate grid. The plates were incubated at 37°C overnight to allow bacterial growth.

Liberation of DNA from bacterial colonies was achieved using the method developed by Grunstein and Hogness (1975) and detailed by Sambrook et al. (1989) with a few modifications. Following treatment with SDS, membranes were treated once with denaturing solution for 10 minutes and twice with neutralizing solution (3 M NaCl, 0.5 M Tris-HCl, pH 7.5) for 5 minutes. Membranes were submerged in 2X SSC and the cell debris was gently rubbed off of the membranes. The membranes were placed between thick chromatography paper (Fisher Scientific) to soak up excess liquid and then the damp membranes were placed in a Bio-Rad GS Gene Linker™ set on program C3 (150 mJoules) to UV-crosslink the DNA to the membranes. Membranes were stored in Saran Wrap at room temperature or used immediately for DNA hybridization.

Prehybridization of membranes involved placing membranes into deep Pyrex® Petri dishes (4 membranes per dish) containing 20 mL hybridization solution (3X SSC, 4X Denhardt's solution, 100 μg/mL denatured salmon sperm DNA) (Sambrook et al. 1989). The dishes were incubated in a shaking water bath (with a lead donut holding the lid down) for 4 hours to overnight at an appropriate temperature (45-65°C). The hybridization temperature for each specific probe was determined by subtracting 25°C from the probe melting temperature (T_m). The T_m value was calculated using the formula: $T_m = 81.5°C + 16.6\log M + 0.41(\%G+C) - 500/n - 0.61\%$ formamide) where M is the ionic strength (0.45 for 3X SSC) and n is the shortest region of homology (Hopwood et al. 1985). Radiolabeled probe DNA (4×10^6 cpm) was added to the hybridization solution and incubation at the appropriate hybridization temperature was continued overnight. Membranes were transferred to rubber-lid Pyrex® glass baking dishes and washed twice with 2X SSC + 0.1% SDS (w/v) for 30 minutes and twice with 0.2X SSC + 0.1% SDS (w/v) for 30 minutes. All washes were done in the shaking water bath at the same hybridization temperature. Membranes were blotted between paper towels to remove excess liquid and wrapped in Saran Wrap. The membranes were then exposed to a Fuji phosphor screen for 30 minutes – 2 hours and the screen was scanned using the Fuji FLA5000 scanner located in MBSU.

2.2.7.2. *Streptomyces* colony blot hybridization

Large scale screening of *Streptomyces* transformants harbouring recombinant plasmids was achieved following the colony hybridization method detailed by Kieser et al. (2000) with a few modifications. An ethanol-proof pen was used to draw a grid onto Whatman® 541 filter paper discs (70 mm) to accommodate 24 *Streptomyces* patches and the grids were autoclaved covered in aluminum foil. The filter discs were overlaid onto solid R2YE medium containing the appropriate antibiotic for plasmid selection. An acetate grid was taped onto the back of the agar side of another R2YE plate containing the appropriate antibiotic. Sterile toothpicks were used to replica patch *Streptomyces* transformants first to the R2YE plate containing the filter disc and second to the R2YE plate with the taped acetate grid. The plates were incubated for 3-5 days at 30°C until dense patches of growth appeared. Treatment of filter discs and subsequent hybridization were performed as described by Kieser et al. (2000). Following washes with hybridization solution, filter discs were washed twice with 2X SSC for 30 minutes and twice with 0.2X SSC for 45 minutes. The membranes were exposed to a Fuji phosphor screen and scanned as described in section 2.2.7.1.

2.2.8. Radiolabeling of DNA

DNA fragments larger than 200 bp were radiolabeled using the random primed DNA labeling kit (Roche) following the manufacturer's directions. The reaction mixture contained 9 μL denatured DNA, 2 μL hexanucleotide mix, 3 μL dNTP mix (1.5 mM each of dATP, dGTP, and dTTP), 5 μL [α ^{32}P]dCTP (10 μCi/μL; Perkin-Elmer), and 1 μL Klenow enzyme (2 units/μL). The reaction mixture was incubated either at room temperature overnight or at 37°C in a water-filled temperature block for 1-6 hours. The labeled DNA fragment was purified away from unincorporated dNTPs using Micro Bio-Spin® 6 chromatography columns (Bio-Rad) according to the manufacturer's directions. The degree of labeling was assessed by counting 1 μL of the labeled DNA preparation using the ^{32}P-Cerenokov program in a Beckman LS 3801 scintillation counter. Labeled DNA was used immediately or stored at -20°C.

DNA fragments smaller than 200 bp were radiolabeled at the 5' end in a 10 µL reaction volume containing 1-2 pmoles DNA, 1X polynucleotide kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT), 50 µCi [γ ^{32}P]ATP (Amersham Biosciences), and 1 unit polynucleotide kinase (Roche). The reaction mix was incubated at 37°C in a water-filled temperature block for 30 minutes. An additional 1 unit polynucleotide kinase was added to the reaction and incubation at 37°C continued for another 30 minutes. The reaction volume was diluted to 50 µL with mQH_2O and labeled DNA was precipitated on ice for 30 minutes with 2 volumes 95% ethanol, 1/10 volume 3 M NaOAc (pH 5.2), and 20 µg glycogen. The precipitated DNA was recovered by centrifugation, washed with 80% ethanol, and redissolved in mQH_2O. The degree of radiolabeling was assessed as described above and used immediately or stored at -20°C.

2.2.9. Construction of a markerless *ΔbldD* mutant

Creation of a markerless *ΔbldD* mutant was achieved using the REDIRECT© technology according to the directions of the supplier (B. Gust; Plant Bioscience Ltd., Norwich, UK) (Gust et al. 2003). Details of the procedure are further described later in Results section 3.1.2.

2.3. RNA methods

2.3.1. RNA isolation

Isolation of *Streptomyces* RNA followed a modified version of the Kirby et al. (1967) procedure, which is described by Hopwood et al. (1985), with several changes. To prevent induction of cold shock proteins, all steps pertaining to cell harvesting prior to cell lysis were performed at room temperature. To prevent RNA digestion by RNases, all steps after cell lysis were performed with gloves, DEPC-treated glassware and reagents (where appropriate), and RNase-free tubes and micropipettor tips. For solid medium time courses, R2YE plates overlaid with cellophane discs were inoculated with the appropriate strains and incubated at 30°C. At various time points post-inoculation, the biomass from the plates was scraped into Universals containing 5 mL 1X Kirby's mix with ~2 cm glass beads (3 mm diameter; Fisher). For induction time courses, strains were grown in liquid medium, induced and the RNA harvested at various time points post-induction. For each strain, six 50 mL R2YE + 0.001% antifoam (v/v) broth cultures were grown at 30°C on a floor shaker (240 rpm) until mid-exponential phase growth (judged visually) was reached. The cultures were pooled into a 1 L flask, mixed, and 50 mL volumes were realiquoted to 250 mL flasks containing spring coils. One flask was left as the uninduced control, while thiostrepton was added to the other 5 flasks to a final concentration of 30 µg/mL. The flasks were returned to the floor shaker and removed after 15, 30, 45, 60, and 120 minutes for RNA isolation. Each culture was transferred into 2 Universals and centrifuged for 2 minutes at 3000 rpm. The cells were washed with mQH_2O and combined into 1 Universal. To each cell pellet, 5 mL 1X Kirby's mix and ~2 cm glass beads were added.

Cells were lysed in the 1X Kirby's mix by agitating samples on a vortex mixer for 2 minutes, after which time, the samples were placed on ice. Samples were mixed with 5 mL Tris-buffered phenol:chloroform:isoamyl alcohol (25:24:1), transferred to 13 mL (16 mm x 100 mm) round bottom polypropylene tubes (Sarstedt), and the phases were separated by centrifuging samples for 10 minutes at 8500 rpm and 4°C in an SS34 rotor of a Sorvall® Evolution RC centrifuge. The upper aqueous phase in each tube was extracted 2-3 more times with 5 mL phenol:chloroform:isoamyl alcohol until there was virtually no interface between the organic and aqueous phases. Nucleic acid in each aqueous phase was precipitated with 1 volume isopropanol and 1/10 volume 3 M NaOAc (unbuffered) at -86°C until samples were frozen. Following thawing and centrifugation of samples, the precipitated nucleic acid was washed with 2 mL 95% ethanol, air dried, redissolved in 450 µL RNase-free water (Gibco-BRL), and transferred to 1.5 mL polypropylene microcentrifuge tubes. DNA in each sample was digested by treatment with 70 units RNase-free DNase I (Roche) in 1X DNase I buffer at room temperature for 30 minutes. A second aliquot of 70 units RNase-free DNase I was added to each sample and incubation at room temperature continued for an additional 30 minutes. The samples were extracted 2-3 times with 500 µL phenol:chloroform:isoamyl alcohol followed by 2 extractions with 500 µL chloroform:isoamyl alcohol (24:1). RNA in the aqueous phase was precipitated with isopropanol and 3 M NaOAc as described above. Precipitated RNA was rinsed with 80% ethanol, air dried, and redissolved in 100 µL RNase-free water. A dilution of each RNA sample was made and the approximate RNA concentration was determined by reading the absorbance at 260 nm using a UV spectrophotometer (Ultraspec 3000; Pharmacia Biotech). The quality of RNA was determined by analyzing 5-15 µg on a 1.4% agarose gel in a 1X TBE buffer system (see section 2.2.2.1). If the RNA was found to be intact and free of DNA contamination, 1 volume isopropanol and 1/10 volume 3 M NaOAc were added before storing RNA samples at -86°C. Correction of the RNA concentration was accomplished by northern hybridization probing for 16S rRNA (see below in section 2.3.2).

2.3.2. Northern hybridization

Northern hybridization was performed as described by Williams and Mason (1985). In 1.5 mL polypropylene microcentrifuge tubes, 10 μg RNA that had been precipitated and redissolved in 2.5 μL RNase-free water, was mixed with 2 μL deionized 6 M glyoxal, 1.5 μL 80 mM NaH_2PO_4-Na_2HPO_4, and 6 μL DMSO. The samples were incubated in a 50°C water bath for 1 hour and placed on ice for 5 minutes. To each sample, 3 μL RNA loading dye [98% deionized formamide (v/v), 10 mM EDTA, 0.025% xylene cyanol (w/v), 0.025% bromophenol blue (w/v)] was added and the samples were resolved on a 1.25% agarose gel in recirculating 10 mM NaH_2PO_4-Na_2HPO_4 buffer for ~4.5 hours at 58 V until the dye front was 2/3 distance from the wells. The RNA in the gel was transferred to Hybond™ nylon membrane overnight using the capillary elution method described by Sambrook et al. (1989). The RNA was UV-crosslinked to the membrane as described in section 2.2.7.1 and then the membrane was dried for 1 hour at 80°C under vacuum to remove the glyoxal. The membrane was placed into a glass hybridization bottle (Fisher) containing 10 mL hybridization solution and incubated in a hybridization oven (Fisher) at the appropriate temperature for 3-6 hours before the addition of radiolabeled (2 X 10^6 cpm) BKL54 primer (for detection of 16S rRNA; see Table 2.3) followed by continued incubation at the same temperature overnight (see section 2.2.7.1). Subsequent washes and exposure of the membrane to the Fuji phosphor screen were performed as described in section 2.2.7.1. Following scanning of the phosphor screen, RNA concentration was corrected using the ImageQuant software of the FLA-5000 Imaging System.

2.3.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized using the SuperScript™ III first-strand synthesis kit (Invitrogen). In a 0.2 mL PCR tube (Rose Scientific Ltd.), the following was added: 5 μL RNA (0.2 μg/μL), 1 μL random hexamers (50 ng/μL; Invitrogen), 1 μL dNTPs (10 mM), 1 μL DMSO, and 5 μL RNase-free water. This mixture was heated to 65°C in a Biometra® T personal thermocycler and cooled on ice for at least 2 minutes. Once the tube was cooled, 4 μL first-strand buffer (5X), 1 μL DTT (0.1 M), 1 μL RNase Out™ (40 units/μL), 0.5 μL RNase-free water, and 0.5 μL Superscript™ III (200 units/μL) were added. For a control containing no reverse transcriptase, the reaction included 0.5 μL RNase-free water in place of 0.5 μL SuperScript™ III. The reaction was incubated at 25°C for 15 minutes, 55°C for 1 hour, and 70°C for 15 minutes. The reaction (referred to as the RT reaction) was then used as template for PCR.

Each RT reaction (1-10 μL) was used as template in a 50 μL PCR reaction containing 1X Taq buffer [50 mM Tris-Cl, pH 9.0, 1.5 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, 0.1 mg/mL BSA (non-acetylated), 10 mM NH_4SO_4, 0.2 mM dNTPs], 1 M betaine, 25 pmoles of each primer, and 2.5 units in-house Taq polymerase. Occasionally, smaller scale PCR reactions were performed with all reaction components adjusted for a 25 μL final volume. The PCR reaction was cycled at 96°C for 2 minutes followed by 20-28 cycles of 96°C for 30 seconds, 62°C for 30 seconds, and 68°C for 2 minutes then finally at 68°C for 5 minutes. PCR reaction conditions and cycling parameters were provided by C. Anders (Department of Biological Sciences, University of Alberta). To each PCR reaction, 10 μL (for 50 μL reactions) or 5 μL (for 25 μL reactions) DNA loading dye was added and 15 μL of the resulting mixtures were resolved on a 5% PAG in a 1X TBE buffer system (see section 2.2.2.1).

2.3.4. Real-time RT-PCR

Before real-time RT-PCR experiments could be performed, amplification efficiencies of the primer pairs, LB5-LB6 and hrdBF-hrdBR, had to be compared to determine which method of relative quantification would be most appropriate to quantitate the results of the real-time RT-PCR analyses. To do this, first-strand cDNA was synthesized as described above in section 2.3.3 and the 20 μL RT reaction was mixed with 10 μL mQH_2O. Seven 4-fold serial dilutions were prepared from each 30 μL RT sample. In an optical 96 well Fast thermal cycle plate (Applied Biosystems), 10 μL reactions (performed in triplicate) were done containing 5 μL 2X Platinum® PCR mix [20 mM Tris-Cl, pH 8.3, 100 mM KCl, 6 mM $MgCl_2$, 1.6% glycerol (v/v), 0.02% Tween 20 (v/v), 4% DMSO (v/v), 0.4 mM dNTPs, 2X ROX (Invitrogen), 0.5X SYBR® Green I dye (Invitrogen), 0.06 units/μL Platinum® Taq polymerase (Invitrogen)], 2.5 μL primer mix (1.6 pmole/μL of each primer), and 2.5 μL RNA template (undiluted and 4-fold serial dilutions of RT reaction). The wells were sealed with an optical adhesive cover (Applied Biosystems), mixed on a vortex mixer briefly, and centrifuged for 2 minutes at 1500 rpm in an Eppendorf centrifuge model 5810. The reactions were subjected to the following conditions in a 7500 Fast Real-Time PCR System (Applied Biosystems): (1) 95°C for 2 minutes, (2) 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, and (3) 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Reaction conditions and cycling parameters were provided by T. Locke (MBSU, Department of Biological Sciences, University of Alberta).

2.4. Protein methods

2.4.1. Protein overexpression and purification

2.4.1.1. His$_6$-BldD overexpression and preparation of cell-free extracts from *E. coli*

LB overnight cultures of *E. coli* JM109 harbouring the pQE(BldD)$^+$ plasmid (see Table 2.2) were diluted 1:100 in fresh LB medium containing 100 µg/mL ampicillin and incubated on a floor shaker (220-240 rpm) at 37°C for 3-4 hours until an OD_{600} ~0.5-0.7 was reached. To the culture, IPTG was added to a final concentration of 1 mM and the culture was incubated on a shaker (220 rpm) at room temperature for 16-18 hours or at 37°C for 3-4 hours. *E. coli* JM109/pQE(BldD)$^+$ cells were recovered in Universals by centrifuging for 10 minutes at 3000 rpm and 4°C in an International centrifuge model PR-J (for 10-25 mL cultures) or in 50 mL Nalgene™ centrifuge tubes (Nalge Company) by centrifuging for 5 minutes at 5000 rpm and 4°C in a Sorvall® SS34 rotor (for 50-200 mL cultures). The cell pellets were washed with mQH_2O and resuspended in 0.5-8 mL Tris sonication buffer [50 mM Tris-Cl, pH 8.5, 300 mM NaCl, 1X Complete EDTA-free protease inhibitor cocktail (Roche)]. Small aliquots (1-1.5 mL) of the cell suspension were transferred to 1.5 mL polypropylene microcentrifuge tubes. The cells were lysed by sonication using a Branson sonifier 450 (microprobe, constant duty, setting 1) on ice for 3-5 pulses of 20 seconds with 15-30 second rests on ice between pulses. The samples were centrifuged in a microcentrifuge for 10 minutes at maximum speed and 4°C to pellet cell debris. Small aliquots (50-500 µL) of the supernatant were stored at -86°C.

2.4.1.2. Purification of His$_6$-BldD protein from *E. coli*

Purification of His$_6$-BldD protein followed the nickel affinity purification protocol described by Qiagen with several modifications. A 200 mL culture of *E. coli*

JM109/pQE(BldD)$^+$ was grown and induced as described in section 2.4.1.1. Following cell recovery, the cell pellet was resuspended in ~4-8 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0, 1X Complete EDTA-free protease inhibitor cocktail) to give a thick paste. The cell suspension was transferred to a 13 mL polypropylene tube and the cells were lysed by sonication as described in section 2.4.1.1. The cell lysate was then transferred to 1.5 mL polypropylene microcentrifuge tubes and cellular debris was removed as described in section 2.4.1.1. The supernatant was transferred to fresh 1.5 mL polypropylene microcentrifuge tubes and stored at -86°C or used immediately for binding to Ni-NTA resin (Qiagen).

Prior to mixing with the cell-free extract, 1 mL 50% slurry of Ni-NTA resin was mixed with 4 mL lysis buffer and the resin was allowed to settle for 1 hour at 4°C. The lysis buffer was removed and the equilibrated resin was mixed with 4 mL cell-free extract. The mixture was incubated at 4°C for 1 hour or overnight with gentle mixing on a Labquake® rotisserie (Barnstead/Thermolyne) and then transferred to a 10 mL gravity flow Poly-Prep® chromatography column (Bio-Rad) to allow settling of the resin. After the flow-through was collected, the resin was washed with 5-6 aliquots of 3 mL cold wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, pH 8.0). His_6-BldD protein was then eluted from the column by washing the resin with 4 aliquots of 250 μL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and 2 aliquots of 250 μL elution buffer + 100 mM imidazole. Five percent of each elution fraction was analyzed by SDS-PAGE (see section 2.4.3). Fractions determined to contain abundant and pure His_6-BldD protein were pooled and the total protein content was quantified (see section 2.4.2).

2.4.1.3. Protein overexpression in *S. coelicolor*

Strains harbouring pIJ6902 and its derivatives were cultivated in R2YE medium at 30°C on a floor shaker for 16-24 hours until mid-exponential phase growth (judged visually) was reached. The cultures were then inoculated with thiostrepton to a final concentration of 30 μg/mL and returned to the shaker for 3 hours of induction. Strains harbouring pSH19 and its derivatives were grown in a mixture of 3:2 YEME:TSB (Kieser et al. 2000) containing 30 μg/mL thiostrepton at 30°C on a floor shaker until mid-exponential phase growth was achieved before induction with a final concentration of 1 mg/mL ε-caprolactam (Aldrich) for 16 or 24 hours. Purification of cell-free extracts was performed as described below in section 2.4.1.4.

2.4.1.4. Preparation of cell-free extracts from *Streptomyces*

Preparation of cell-free extracts from liquid-grown cultures involved recovering cells in Universals by centrifuging for 10 minutes at 3000 rpm and 4°C. The cell pellet was washed with mQH_2O and resuspended in 0.5-2 mL Tris sonication buffer (50 mM Tris-Cl, pH 8.5, 300 mM NaCl, 1X Complete EDTA-free protease inhibitor cocktail), HEPES sonication buffer (50 mM HEPES, pH 7.5, 1X Complete EDTA-free protease inhibitor cocktail), or PBS sonication buffer (2.7 mM KCl, 1.8 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, 1X Complete EDTA-free protease inhibitor cocktail). Small aliquots (1-1.5 mL) of the cell suspension were transferred to 1.5 mL polypropylene microcentrifuge tubes. For surface-grown cultures, the biomass was scraped off the cellophane discs overlaid on R2YE plates directly into 1.5 mL polypropylene microcentrifuge tubes. Volumes of 0.5-1 mL of the above listed sonication buffers were added to the tubes and the biomass was resuspended by vortex mixing. Cells were lysed by sonication and the lysate clarified as described in section 2.4.1.1. Small aliquots (50-500 µL) of the supernatant were stored at -86°C.

2.4.1.5. Purification of His$_6$-BldD protein from *Streptomyces*

Purification of His$_6$-BldD protein from *Streptomyces* followed the same procedure described in section 2.4.1.2 for purification of His$_6$-BldD protein from *E. coli*.

2.4.2. Protein quantification

Quantification of protein content followed the Bradford dye-binding method using the Bio-Rad protein assay kit according to the manufacturer's directions. The microassay procedure was followed and bovine gamma globulin (Bio-Rad) was used as the standard.

2.4.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method developed by Laemmli (1970). Protein samples were mixed with an equal volume of 2X SDS-PAGE buffer [100 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 0.29 M β-mercaptoethanol, 0.2% bromophenol blue (w/v)], boiled for 5-10 minutes, and electrophoresed on mini 10-15% SDS polyacrylamide gels [gel recipes based on Laemmli (1970)] in Tris-Glycine-SDS buffer [25 mM Tris-Cl, 192 mM glycine, 0.1% SDS (w/v)] at 200 V until the dye front was near or at the bottom of the gel. Gel electrophoresis used the Bio-Rad Mini-PROTEAN® 3 system. Protein detection was achieved by either Coomassie Brilliant Blue staining (section 2.4.4) or Western analysis (section 2.4.5). For size determination, either SDS-PAGE broad range molecular weight standards (Bio-Rad), Kaleidoscope prestained standards (Bio-Rad), or PageRuler™ prestained protein ladder (Fermentas Life

Sciences) was used. Prestained protein ladders were always used when Western analysis followed gel electrophoresis.

2.4.4. Coomassie Brilliant Blue staining

Following electrophoresis as described in section 2.4.3, the gel was placed into a Pyrex® glass dish containing 0.1-0.25% Coomassie Brilliant Blue R250 stain [Coomassie powder dissolved in mQH_2O with 10% acetic acid (v/v) and 45% methanol (v/v)] and incubated with gentle agitation on a rocking platform for 45 minutes at 37°C or for 4 hours to overnight at room temperature. The gel was then destained in Coomassie Brilliant Blue destaining solution [10% acetic acid (v/v), 20% methanol (v/v)] with gentle agitation on a rocking platform at room temperature for 1 full day. Kimwipes were placed in the dish with the gel to soak up excess stain.

2.4.5. Western immunoblot analysis

Following electrophoresis as described in section 2.4.3, the gel was soaked in transfer buffer [19.2 mM Tris-Cl, pH 8.0, 192 mM glycine, 20% methanol (v/v), 0.015% SDS (w/v)] for 30 minutes at room temperature with gentle agitation. At the same time, a piece of Immuno-Blot™ PVDF membrane (Bio-Rad) cut to the size of the gel was rinsed with 100% methanol and equilibrated in transfer buffer for 15 minutes with gentle agitation. Western transfer used the Bio-Rad Mini Trans-Blot® system and the transfer apparatus was assembled according to the manufacturer's directions. The transfer was done for 1 hour at 4°C and 350-400 mA. The membrane was then rinsed 3 times with mQH_2O for 5 minutes each time with agitation on a rocking platform. Following a final rinse with wash buffer [20 mM Tris-Cl, pH 7.6, 140 mM NaCl, 0.125% Tween® 20 (v/v)], the membrane was incubated in 20 mL 10% skim milk solution (w/v) at room temperature overnight with gentle agitation. Ten millilitres of the skim milk solution were removed and αBldD antibodies were diluted 1:20000 (or 1:1000 when affinity-purified αBldD antibodies was used) in the remaining 10 mL skim milk solution followed by continued incubation of the membrane in the skim milk/antibodies mixture at room temperature with gentle agitation for 1 hour. The membrane was rinsed by agitating in wash buffer: 2 quick washes, 2 washes for 5 minutes, and 1 wash for 15 minutes. The membrane was incubated in 10 mL skim milk solution with a 1:10000 dilution of donkey anti-rabbit Ig, horseradish peroxidase-linked secondary antibody (Amersham Biosciences) at room temperature with gentle agitation for 1 hour. The membrane was rinsed with wash buffer as described above then agitated for 1 minute in a mixture of 1 mL enhanced luminol reagent and 1 mL oxidizing reagent (Western Lightning™ Chemiluminescence Reagent Plus; Perkin-Elmer Life Sciences). Thick chromatography paper was used to blot away excess liquid and the membrane was wrapped in Saran Wrap and taped face up in an X-ray cassette. The membrane was exposed to X-ray film (BioMax XAR scientific imaging film; Kodak) in the dark and the film developed using a FUJI RGII X-ray film processor.

2.4.6. Preparation of affinity-purified αBldD antibodies

Cyanogen bromide activated Sepharose 4B powder was purchased from Amersham Biosciences and the powder was swelled according to the manufacturer's recommendations. The powder was placed in the center of a sintered glass filter and the filter was placed on top of a vacuum flask. The powder was swelled (0.3 g swells to ~1 mL) by mixing it with ~75 mL 1 mM HCl. The acid was added in several aliquots and the acid was allowed to drip through the filter to be collected in the flask below. The speed of acid draining was monitored and adjusted to allow the 75 mL acid to drain in ~15 minutes. The swelled resin was scraped into a 13 mL round bottom polypropylene tube. The filter was rinsed with some additional 1 mM HCl and this liquid was added to the resin in the tube. The resin was recovered by centrifuging for a few seconds at 3000 rpm in an International centrifuge model PR-J. The acid was discarded and the resin washed twice more with 5 mL coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3). The ~1 mL swelled resin was mixed with 2 mL His_6-BldD protein (~2-2.5 mg) suspended in coupling buffer. The mixture was incubated at 4°C overnight with gentle rotation on a Labquake® rotisserie. The resin was collected, the supernatant discarded, and the resin mixed with blocking buffer (1 M ethanolamine) and incubated for 2 hours at room temperature. The resin was then collected and washed 3 times each alternating with coupling buffer and acetate buffer (0.5 M NaCl, 0.4 M acetate, pH 4.0). The resin was used immediately for antibody binding or stored at 4°C in 2 volumes PBS buffer (Sambrook et al. 1989) containing 0.01-0.02% sodium azide preservative (v/v).

Eight to ten millilitres crude αBldD serum were diluted in cold PBS buffer to a volume of 100 mL in a glass beaker. The solution was mixed at a moderately fast speed on a magnetic stirrer and ammonium sulfate powder was added slowly to 40% saturation. The mixture was left mixing for 1 hour at 4°C followed by transfer to clear 50 mL Nalgene™ centrifuge tubes (Nalge Company) and centrifugation for 15 minutes at 5000 rpm and 4°C in a Sorvall® SS34 rotor. The supernatant was discarded, and the pellets were redissolved in a total volume of 2.5 mL PBS buffer. The precipitated IgG was dialyzed (Spectra/POR® molecular porous membrane tubing, 12000-14000 MWCO, 15.9 mm diameter; Spectrum medical industries, Inc.) against 1 L cold PBS buffer for 1 hour and then against 4 L cold PBS buffer at 4°C overnight with buffer stirring. The IgG was transferred to a 13 mL polypropylene tube and centrifuged for 5 minutes at 3000 rpm and 4°C in an International centrifuge model PR-J to remove particulate debris. The IgG was used immediately or stored at 4°C in 0.02% sodium azide (v/v).

Approximately 23 mg of the ammonium sulfate-precipitated IgG was diluted to 2 mL with PBS buffer and mixed with 1 mL cyanogen activated Sepharose 4B coupled to His$_6$-BldD as mentioned above. This mixture was incubated at 4°C overnight with gentle rotation on a Labquake® rotisserie. The resin was collected by centrifugation at 3000 rpm for a few seconds. The supernatant was saved to allow for determination of the percent antibody capture. The resin was washed 3 times with 5 mL cold PBS buffer and the antibody was eluted with four 500 μL aliquots of 0.1 M glycine (pH 2.0). Each elution involved incubating at room temperature for 10 minutes with occasional mixing, centrifuging a few seconds at 3000 rpm to collect resin, and transferring the supernatant to 1.5 mL polypropylene microcentrifuge tubes containing 50-100 μL 2 M Tris-Cl (pH 8.0) which allowed for neutralization of the antibody solution. The elution fractions were combined and dialyzed against 500 mL cold PBS buffer for 1 hour and then against 2 L cold PBS buffer at 4°C overnight with buffer stirring. The antibody preparation was transferred to a 13 mL polypropylene tube and 100% glycerol was added to give a final concentration of 10% glycerol (v/v). Following quantification of the antibody preparation, the preparation was divided into 25-200 μL aliquots and stored at -86°C.

2.4.7. Coupling antibodies to protein A Sepharose

Affinity-purified αBldD antibodies were coupled to protein A Sepharose (Sigma-Aldrich) using a protocol supplied by the Stuart lab (Department of Biochemistry, University of Alberta). Protein A Sepharose (75 mg) was mixed with 1 mL PBS buffer in a 1.5 mL polypropylene microcentrifuge tube and allowed to swell for 10 minutes at room temperature. The swelled resin was washed twice with 1 mL PBS buffer then mixed with 300 μL (=150 μg) affinity-purified αBldD antibodies. The resin/antibodies mixture was incubated at 4°C overnight on a Labquake® rotisserie then washed twice with 10 bed volumes 0.2 M sodium borate (pH 9.0). The resin was then resuspended in 1 bed volume 0.2 M sodium borate (pH 9.0) and a 20 μL aliquot was removed to save as an uncrosslinked sample. The volume of the remaining resin was adjusted to 1 mL with PBS buffer and mixed with 500 μL 30 mM DMP crosslinker (Pierce). The resulting mixture was incubated at room temperature for 25 minutes on a Labquake® rotisserie and the crosslinking reaction was quenched by washing the resin twice with 1 mL 0.2 M ethanolamine (pH 8.0). The resin was then resuspended in 1 mL 0.2 M ethanolamine (pH 8.0) and incubated at room temperature for 2 hours on a Labquake® rotisserie. The resin was washed twice with 1 mL PBS buffer and finally resuspended in 1 bed volume PBS buffer. A 20 μL aliquot of the resuspended resin was removed to save as a crosslinked sample. The antibody-coupled resin was stored at 4°C with 0.02% sodium azide preservative (v/v). To check the coupling efficiency, the uncrosslinked and crosslinked samples were each mixed with 20 μL 2X non-reducing SDS-PAGE buffer [100 mM Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 0.2% bromophenol blue (w/v)] and boiled for 5 minutes. Following brief centrifugation to pellet the resin, the supernatant from each sample was applied onto a mini 10% SDS-PAG and analyzed by SDS-PAGE (see section 2.4.3).

2.4.8. Immunoprecipitation (IP)

The immunoprecipitation technique was based on the procedure described by Jakimowicz et al. (2002). In a 1.5 mL polypropylene microcentrifuge tube, the following was combined: 1-3 mg cellular extract proteins (diluted to 200 μL with PBS sonication buffer when required), 500 μL IMP buffer [50 mM Tris-Cl, pH 7.5, 300 mM NaCl, 1 mg/mL BSA, 2% Nonidet P40 (v/v), 1X Complete EDTA-free protease inhibitor cocktail], and 5-10 μg affinity-purified αBldD antibodies. The IP reaction mix was incubated at 4°C overnight with gentle rotation on a Labquake® rotisserie. Following overnight incubation, 40 μL 50% protein A Sepharose slurry was added to each reaction and incubation continued for 2-6 hours. The resin was collected by centrifuging in a microcentrifuge for 2 minutes at 3000 x g and 4°C. The supernatant was discarded and the resin was washed 4-6 times with 1 mL IMP wash buffer [50 mM Tris-Cl, pH 7.5, 300 mM NaCl, 0.5% Nonidet P40 (v/v), 0.1% SDS (w/v)]. After the last wash, a gel loading tip was used to remove as much liquid as possible and the resin was resuspended in 20 μL 2X non-reducing SDS-PAGE buffer. The resin was boiled for 5 minutes at 95°C to elute the immunocomplexes off the protein A Sepharose beads. The supernatant containing the eluted immunocomplexes was collected following brief centrifugation in a microcentrifuge at 12000 x g to pellet the beads. The supernatant was analyzed by SDS-PAGE (see section 2.4.3).

For large scale IP experiments, 10-12 IP reactions were performed for each cell-free extract sample and the immunocomplexes were eluted from the resin by boiling each reaction in 10 μL 2X non-reducing SDS-PAGE buffer. The supernatants from the 10-12 IP reactions were pooled and loaded into 1 well of a large 10% or 12% SDS-PAG. The

IP sample was electrophoresed at 200 V for 5-6 hours in a PROTEAN® II xi electrophoresis unit (Bio-Rad) with the attached cooling system set at 12.5°C to maintain the buffer temperature at room temperature.

When IP samples needed to be concentrated for two-dimensional polyacrylamide gel electrophoresis (see below in section 2.4.9), the resin, after the washes, was resuspended in 2% CHAPS (w/v) or mQH_2O, boiled for 5 minutes, and centrifuged at 12000 x g. The supernatant was collected and concentrated to 0-10 μL in a heated vacuum centrifuge (SPD Speed Vac® with UVS400 Universal Vacuum System; Thermo Savant).

2.4.9. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

In 1.5 mL polypropylene microcentrifuge tubes, 100 or 400 μg cellular extract proteins, IP sample, or 5 μL 2D protein molecular weight marker mix (Pierce or Bio-Rad) were each mixed separately with 1.15X rehydration buffer [9.2 M urea, 2.3% CHAPS (w/v), 0.575% Pharmalyte (v/v) (pH 5-8; Amersham Biosciences), 0.0023% bromophenol blue (v/v), 46 mM DTT] to give a 1X rehydration buffer concentration [8 M urea, 2% CHAPS (w/v), 0.5% Pharmalyte (v/v), 0.002% bromophenol blue (v/v), 40 mM DTT] in a final volume of 120-240 μL. Sample loading of isoelectric focusing strips followed the method of rehydration loading according to the directions provided by Amersham Biosciences. Briefly, the protein sample/rehydration buffer mixtures were used to reswell Immobiline® DryStrip gels (7 cm, pH 4-7; Amersham Biosciences) at room temperature overnight. The first dimension was typically run for 2.5-6 hours in the Ettan™ IPGPhor II isoelectric focusing unit (Amersham Biosciences) until 8000-14000 total volt hours were achieved. Typical running conditions consisted of: (1) 500 V for 30 minutes, (2) 1000 V for 30 minutes, and (3) 5000 V for 1.5-5 hours. The strips were placed into 15 mL (16 mm x 125 mm) round bottom polystyrene culture tubes and frozen at -86°C or subjected to SDS-PAGE immediately. Prior to second dimension separation, the strips were equilibrated with 5-10 mL equilibration buffer [50 mM Tris-Cl, pH 8.8, 30% glycerol (v/v), 6 M urea, 2% SDS (w/v), 0.002% bromophenol blue (w/v)] + 10 mg/mL DTT and equilibration buffer + 25 mg/mL iodoacetamide (Bio-Rad) by incubating the strips in the buffers for 15 minutes at room temperature with gentle agitation. The strips were rinsed with Tris-Glycine-SDS protein running buffer then placed on top of mini 15% SDS-PAGE gels (no stacking gel). A ~50 µL 0.5% agarose block containing 5 µL prestained protein molecular weight marker (Fermentas Life Sciences) was placed on one side of each strip. The strips were then overlaid with ~500 µL agarose sealing solution [0.5% agarose (w/v), 0.002% bromophenol blue (w/v), 25 mM Tris-Cl, 192 mM glycine, 0.1% SDS (w/v)]. The gels were run at 200 V in Tris-Glycine-SDS buffer until the dye front reached the bottom of the gels. Detection of protein spots was accomplished by Coomassie Brilliant Blue staining (section 2.4.4) or Western analysis (section 2.4.5).

2.4.10. Large format 2D-PAGE

An aliquot of cell-free extract containing 900 µg total protein content was mixed with 1.15X rehydration buffer to a total volume of 340 µL. Fifteen microlitres 2D protein molecular weight marker mix were combined with 325 µL 1.15X rehydration buffer. The mixtures were used to rehydrate Immobiline® DryStrip gels (18 cm, pH 4-7; Amersham Biosciences) according to the manufacturer's directions. The first dimension was run for 6 hours (~32000 total volt hours) in the Ettan™ IPGPhor II isoelectric focusing unit under the following conditions: (1) 500 V for 30 minutes, (2) 1000 V for 90 minutes, and (3) 8000 V for 4 hours. The strips were placed into glass test tubes and frozen at -86°C or subjected to SDS-PAGE immediately. The strips were equilibrated with 10 mL equilibration buffer + 10 mg/mL DTT and 10 mL equilibration buffer + 25 mg/mL iodoacetamide as described in section 2.4.9. The strips were rinsed with Tris-Glycine-SDS buffer then placed on top of a large 12.5% SDS-PAG. On one side of the strip, a 100 μL 0.5% agarose block containing 10 μL prestained protein ladder was placed, and finally the strip was overlaid with ~2 mL agarose sealing solution. The gel was run in an Ettan™ Dalt Six electrophoresis unit (Amersham Biosciences) at 2.5 watts per gel for 30 minutes and 100 watts total for ~4 hours until the dye front was 1 inch from the bottom of the gel. To maintain the buffer temperature at 25°C, the electrophoresis unit was attached to a cooling system set at 10°C. Visualization of protein spots was achieved by Coomassie Brilliant Blue staining (section 2.4.4).

2.4.11. Protein-protein crosslinking

Streptomyces cultures (100-150 mL) were grown until mid-exponential phase growth (judged visually) was reached. The cells were collected and resuspended in 10 mL P buffer + 1 mg/mL lysozyme (Kieser et al. 2000). The protoplasts were prepared as described in section 2.2.5.5. The protoplasts were lysed in 2-3 mL crosslinking buffer [50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 10% glycerol (v/v), 0.5 mg/mL BSA, 150 mM NaCl, 1 mM DTT]. The cell lysate was divided into 922 μL aliquots and each aliquot was mixed with 40 μL 25X Complete EDTA-free protease inhibitor cocktail, 20 μL DNase I (20 units/μL; Roche), and 10 μL of either mQH_2O or 100 mM BS^3 (Sigma) or EGS (Pierce) crosslinkers. The reactions were incubated for 1 hour in a 30°C water bath before quenching with the addition of 10 μL 0.5 M lysine and incubation for 15 minutes at room temperature with gentle agitation on a rocking platform. The reactions were centrifuged in a microcentrifuge for 10 minutes at maximum speed and 4°C and the supernatants were transferred to new 1.5 mL polypropylene microcentrifuge tubes. The crosslinked samples and uncrosslinked control were stored at -86°C in 100-500 μL aliquots. Western analysis (section 2.4.5) was performed to determine the presence of BldD-dependent complexes.

2.4.12. Sample preparation for mass spectrometric analysis

To prevent contamination from keratin, all samples, reagents, and equipment used for the experiment were handled with gloves. All solutions used were filtered through 0.45 μm filters. All glassware used was treated with 0.1 N HCl for 1-2 hours and thoroughly washed with mQH_2O. Following gel electrophoresis, staining, and destaining, gel slices were excised using new scalpels. The gel slices were placed into 1.5 mL polypropylene microcentrifuge tubes, hydrated with 100 μL sterile water, and stored at 4°C or immediately brought to the Institute for Biomolecular Design (Medical Sciences Building, University of Alberta) for mass spectrometric analysis.

2.5. Protein-DNA analysis

2.5.1. DNA affinity chromatography

To immobilize target DNA onto a column for isolation of BldD protein, the μMACS streptavidin kit (Miltenyi Biotec) was used. Biotinylation of target DNA was accomplished by PCR amplification. For each primer pair, one primer was synthesized with the addition of one biotin unit on the 5' end. The *bldD* promoter fragment was generated by using the primer pair LBU2 and MAE4 giving a 106 bp product while the *sigQ* promoter fragment was generated by using either the primer pair LBU1 and CGA52 to give an 83 bp product or the primer pair LBU1 and CGA41 to give a 170 bp product.

The binding reaction, which was done in a 1.5 mL polypropylene microcentrifuge tube, contained 2.5-15 mg cell-free extract proteins, 6-10 μg biotinylated DNA, 50-100 μg poly dI-dC, 10% glycerol (v/v), 2 mM DTT, 150 mM NaCl, and 10 mM Tris-Cl (pH 8.5). The reaction was incubated at room temperature for 1.5-3 hours with gentle rotation on a Labquake® rotisserie followed by addition of 100 μL streptavidin microbeads and continued incubation for an additional 15-30 minutes. The μMACS column was placed in the μMACS magnetic separator and equilibrated with 200 μL protein equilibration buffer included in the kit and then rinsed with 200 μL 1X binding buffer (10 mM Tris-Cl, pH 8.5, 150 mM NaCl, and 2 mM DTT) containing 10% glycerol (v/v). The entire reaction mix was applied onto the column and the flow-through collected in a 1.5 mL polypropylene microcentrifuge tube. The column was washed with 500 μL volumes of the following buffers containing 10% glycerol (v/v): (1) 1X binding buffer, (2) 1X binding buffer + 100 mM NaCl, (3) 1X binding buffer + 350 mM NaCl, (4) 1X binding buffer + 600 mM NaCl, and (5) 1X binding buffer + 850 mM NaCl. The column was removed from the magnetic separator and washed once more with 1X binding buffer + 10% glycerol (v/v) + 850 mM NaCl. All wash fractions were collected in 1.5 mL polypropylene microcentrifuge tubes. With exception to the flow-through, all fractions were concentrated to 5-15 μL using Microcon® centrifuge columns (MWCO 10000; Millipore). The concentrated samples along with 10 μL of the flow-through and 5 μg purified His$_6$-BldD (expressed in *E. coli*) were each mixed separately with an equal volume of 2X SDS-PAGE buffer and boiled for 10 minutes. The samples were analyzed by SDS-PAGE (section 2.4.3) and the gel stained with Coomassie Brilliant Blue (section 2.4.4).

2.5.2. Chromatin immunoprecipitation (ChIP)

The ChIP procedure was developed by making modifications to the methods described by Solomon and Varshavsky (1985), Jakimowicz et al. (2002), and Shin and Groisman (2005). Wild-type M600 and *ΔbldD* FLP 49 mutant strains were grown to high cell density (late exponential) in 30 mL medium containing a mixture of 3:2 YEME:TSB (Kieser et al. 2000). Cells were recovered and washed with 10 mL HEPES solution (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA) then resuspended in 10 mL HEPES solution with 1% formaldehyde (v/v) and incubated for 10 minutes at room temperature with occasional mixing. The crosslinking reaction was quenched by adding 1 mL 10% glycine (w/v) and incubating the mixture for 5 minutes at room temperature with occasional mixing. The cells were recovered by brief centrifugation, washed with 10 mL cold PBS buffer, resuspended in 1 volume PBS sonication buffer, and transferred to 1.5 mL polypropylene microcentrifuge tubes. For an uncrosslinked control, wild-type cells, not subjected to formaldehyde crosslinking, were treated in the same manner as the crosslinked samples. All cell samples were then lysed by sonication using the Branson sonifier 450 (microprobe, constant duty, setting 1) for 3 X 20 second pulses on ice with 20 second rests on ice between pulses. Cellular debris was removed by centrifugation and the cell lysates were stored in small aliquots at -86°C or used immediately in DNA shearing reactions.

Shearing of chromosomal DNA involved the use of the Enzymatic shearing kit purchased from Active Motif. Two hundred fifty microlitres of each sample lysate were transferred to 1.5 mL polypropylene microcentrifuge tubes and heated for 5 minutes in a 37°C water bath. Following incubation, 12.5 µL of a 1:100 diluted solution of enzymatic shearing cocktail was mixed with each sample lysate and incubation in the 37°C water bath was continued for another 20 minutes. The shearing reaction was stopped by addition of 5 µL cold 0.5 M EDTA (pH 8.0) and incubation on ice for 10 minutes. Protein content was determined using the Bio-Rad protein assay kit as described in section 2.4.2. The degree of shearing was determined by analyzing cell lysates on a 1% agarose gel following treatment of 25 µL of each cell lysate with 5 mg DNase-free RNase A for 1-2 hours at 65°C and 10 mg proteinase K (Roche) for 1 hour at 42°C.

Prior to performing the immunoprecipitation reactions, 225 µL of each cell lysate containing sheared DNA was incubated with 40 µL fresh 50% slurry of protein A Sepharose for 2-4 hours at 4°C on a Labquake® rotisserie to remove any DNA non-specifically binding to protein A Sepharose beads. Pre-cleared cell lysate samples containing approximately 1-1.5 mg proteins were used in the immunoprecipitation reactions as described in section 2.4.8. After washing the protein A Sepharose resin with six 1 mL volumes of IMP wash buffer, the resin was washed twice with 1 mL TE buffer. The immunoprecipitated complexes were eluted by incubating the resin in 100 µL elution buffer [50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% SDS (w/v)] for 30 minutes at 65°C. The resin was recovered following centrifugation and the supernatant was collected in a new 1.5 mL polypropylene microcentrifuge tube. To the resin, another 100 µL aliquot of elution buffer was added and the tube incubated for another 15 minutes at 65°C before pelleting the resin. The supernatant was pooled with the supernatant after the first incubation. To the pooled supernatant, 8 µL 5 M NaCl and 1 µL 10 mg/mL DNase-free

RNase A were added and the mixture incubated for 3 hours to overnight at 65°C to reverse crosslinks. For a positive control, 25-50 μL pre-cleared wild-type cell lysate, which had been treated with formaldehyde crosslinker but not subjected to immunoprecipitation, was diluted to 200 μL with elution buffer and treated with RNase A as described for the other samples. Following incubation at 65°C all samples were treated with 2 μL 10 mg/mL proteinase K for 1.5 hours at 42°C. The samples were extracted once with 1 volume Tris-buffered phenol:chloroform:isoamyl alcohol (25:24:1) and once with 1 volume chloroform:isoamyl alcohol (24:1). The aqueous phase was precipitated for 2 hours to overnight at -20°C with 2 volumes 95% ethanol, 1/10 volume 3 M NaOAc (pH 5.2), and 40 μg glycogen. Precipitated DNA was recovered by centrifugation, washed with 70% ethanol, air dried, redissolved in 100 μL TE buffer, and stored at 4°C.

PCR reactions consisted of 1 μL ChIP DNA, 0.2 mM dNTPs, 0.4 μM of each primer, 1X Expand™ High Fidelity buffer 2 containing 1.5 mM $MgCl_2$, 0-6% DMSO (v/v), and 2.5 units in-house Taq polymerase in a 50 μL reaction volume. The reactions were subjected to 5 minutes denaturation at 95°C followed by 24 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 52-60°C, and extension for 1 minute at 72°C, then subjected to a final 5 minutes at 72°C.

CHAPTER 3:

RESULTS

3. RESULTS

3.1. Studies to isolate and characterize modified BldD

During vegetative growth, BldD represses the transcription of a number of developmental sigma factor genes (*bldN*, *whiG*, and *sigH*) (Elliot et al. 2001; Kelemen et al. 2001). Although much of the work to date has only identified genes whose expression is repressed by BldD, the observation that a *bldD* mutant is pleiotropically defective in aerial mycelium formation and antibiotic production suggests that in addition to its role as a repressor, BldD might also function as an activator of genes controlling differentiation and secondary metabolism (Elliot 2000; Elliot et al. 2001). Microarray analysis examining global gene expression in *S. coelicolor* following *bldD* induction revealed that in addition to at least 13 genes that are repressed when *bldD* is induced, some genes appear to be activated by BldD (C. Galibois, unpublished), thus supporting the idea that BldD may function as an activator of gene expression. One of these activated genes, *sigQ*, encodes another sigma factor and DNA-binding studies confirmed that BldD binds to its promoter region (C. Galibois, unpublished).

For morphological differentiation to occur BldD-mediated transcriptional repression of developmental genes must somehow be relieved. Additionally, the onset of morphological differentiation appears to require transcriptional activation of developmental genes, and this process might involve BldD. The factors(s) involved in overcoming BldD repression and the mechanism(s) regulating the switch between the BldD repressor and activator functions still await discovery. Therefore, the objective of this thesis was to elucidate the cellular mechanisms involved in regulating BldD activity. Post-translational modification, which is a common mechanism to regulate the activity of transcriptional regulators, has been hypothesized to play a role in the control of BldD activity (Elliot 2000).

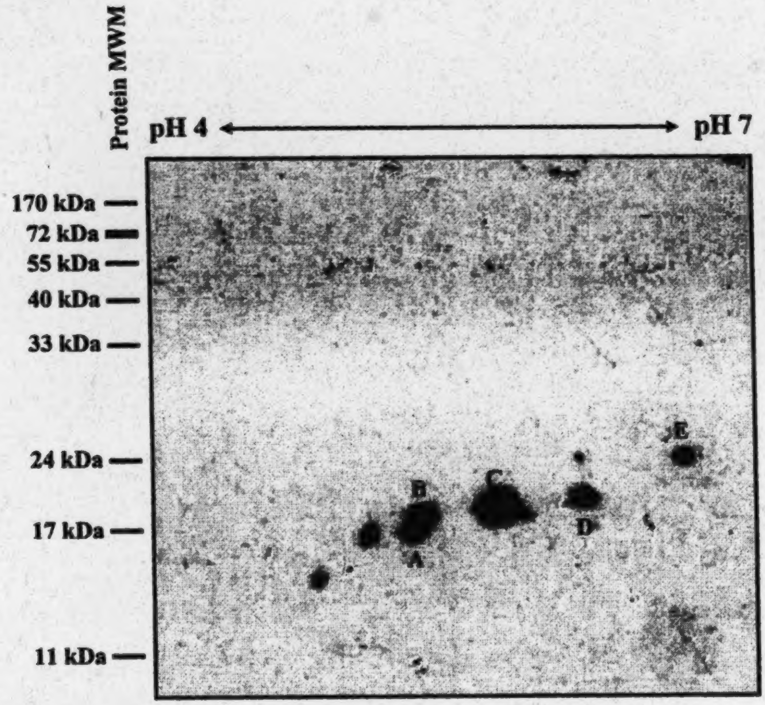
3.1.1. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Previous 2D-PAGE analysis of *S. coelicolor* cell-free extract proteins by A. Hesketh (personal communication) revealed two BldD isoforms (protein identity confirmed by mass spectrometric analysis), each with a distinct expression profile during growth. The major BldD spot, which is expected to be unmodified BldD, is present at all growth time points but begins to decrease as the organism progresses into late-exponential and stationary phase growth. The minor BldD spot, which might represent a modified BldD isoform, is absent from mid-exponential phase growth and does not begin to accumulate until the organism enters late-exponential and early stationary phase growth. In addition to being slightly more basic than the unmodified BldD protein, the putative modified BldD isoform has an apparent molecular weight of ~1 kDa larger than its unmodified counterpart. These findings suggest that BldD may be modified during development, and therefore it was decided to employ the 2D-PAGE technique to analyze *S. coelicolor* cell-free extract proteins prepared from different time points corresponding to the different stages of morphological differentiation. It was hoped that the modified BldD protein, which was expected to accumulate at later time points during growth based on the Hesketh study, could be purified for mass spectrometric analysis to characterize the putative BldD modification. Ultimately, the goal was to address whether the modification was responsible for either controlling the switch between the repressor and activator activities of BldD or overcoming BldD repression of developmental genes at the onset of morphological differentiation.

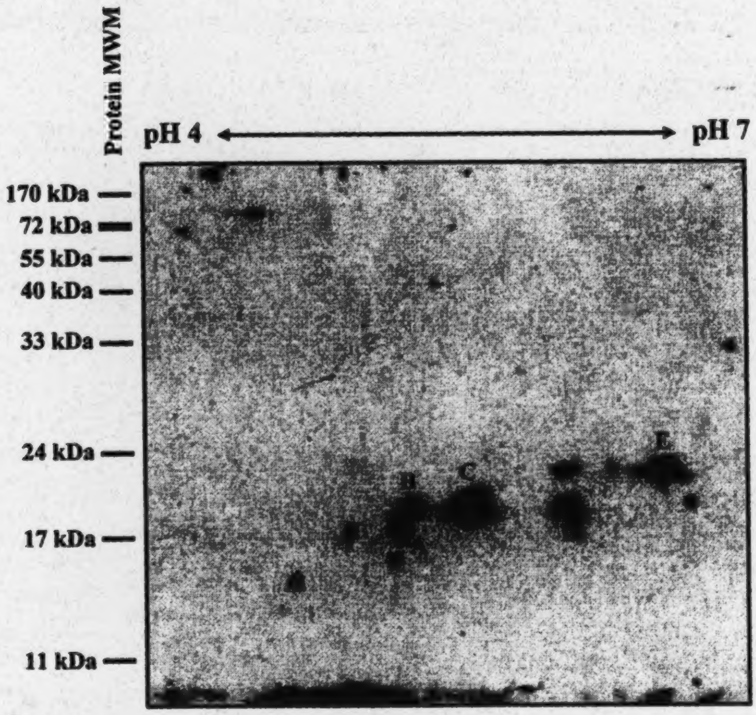
To optimize the 2D-PAGE procedure, cell-free extract (100 µg total protein content) isolated from surface-grown *S. coelicolor* wild-type strain M600 at 30 hours post-inoculation (time point corresponds to aerial hyphae production) was subjected to isoelectric focusing using a 7 cm Immobiline® DryStrip gel (pH 4-7; Amersham Biosciences). Although the Hesketh 2D-PAGE study was performed with cell-free extracts prepared from liquid-grown cultures, the decision to use surface-grown cultures was based on the observation that wild-type *S. coelicolor* does not undergo morphogenesis when submerged in liquid media. Therefore, the use of cell-free extracts from surface-grown cultures might allow for a better correlation between BldD modification and the different morphological states of *S. coelicolor*. Following second dimension separation on a mini 15% SDS-PAG, Western immunoblot analysis was performed to detect the unmodified and modified BldD proteins. Two-dimensional PAGE standards (Bio-Rad) were subjected to first and second dimension separation under the same conditions as the test sample and then stained with Coomassie Brilliant Blue to allow for an estimation of the pI values of the two expected BldD isoforms. Protein molecular weight standards (Fermentas Life Sciences) were included in the second dimension to allow for size estimation of the BldD isoforms.

Surprisingly, instead of the two expected spots corresponding to the BldD isoforms observed by A. Hesketh, Western analysis detected five major (relatively intense) spots (labeled A-E) in the molecular weight size range of unmodified BldD protein and three or four other very minor (faint) spots (Figure 3.1A). The minor spots are most likely nonspecific spots arising from cross-hybridization with αBldD antibody. In considering only the major spots, spot (C), which is the most intense spot, may

(A) 8 M urea


Protein MWM
pH 4
pH 7
170 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
A
B
C
D
E

(B) 9.8 M urea

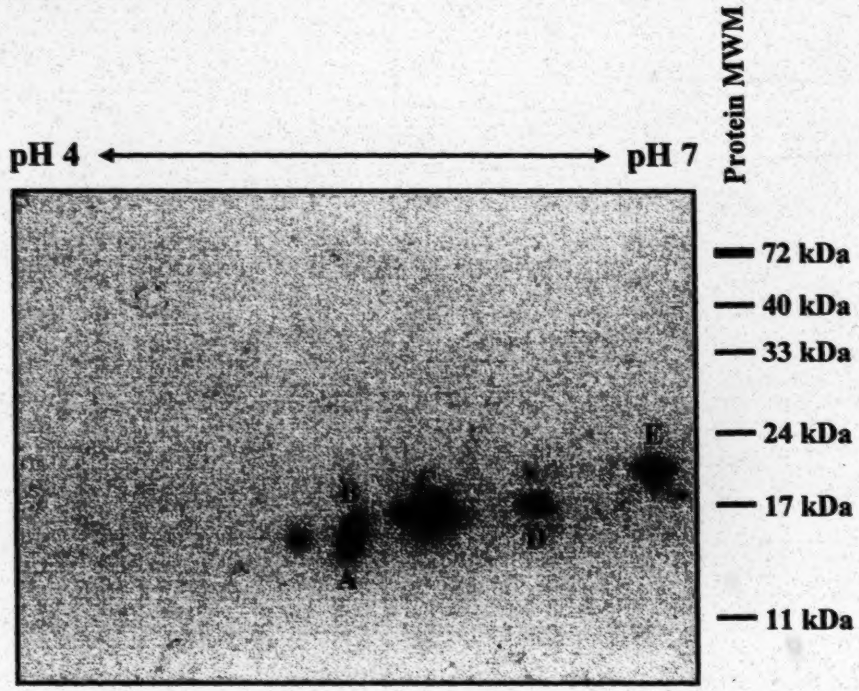

Protein MWM
pH 4
pH 7
170 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
A
B
C
E

represent the unmodified BldD protein because this species appears to be present in greater abundance than the other species. It should be noted, however, that if spot (C) represents the unmodified BldD protein, then the identity of spots (B) and (D) are difficult to assess because while these two spots differ in pI values from spot (C), they both appear to be roughly the same molecular weight size as spot (C). Spot (E), the most basic spot, may also be a good candidate to be the unmodified BldD protein because unmodified BldD, which has a deduced molecular weight of 18.2 kDa, has been observed to migrate closer to the 24 kDa molecular weight marker when electrophoresed on a 15% SDS-PAG. Therefore, if spot (E) represents the unmodified BldD protein, then spots (A), (B), (C), and (D) could each represent a different BldD degradation product. Since the spot corresponding to the unmodified BldD protein could not be clearly defined, it was not possible to determine which spot might correspond to the modified BldD protein. Additionally, because the Coomassie-stained gel of the 2D-PAGE standards did not reveal any spots, the pI values for the spots detected by Western analysis could not be estimated, making it more difficult to predict the identities of the protein spots. The predicted pI value for BldD is 6.8 as determined by PepTool; however, the pI value was calculated to be 6.1 using the method of Mosher et al. (1993) as described by Bignell (2003). Since the pI value varied depending on the formula used, it was important to determine empirically the pI value of BldD.

As an attempt to reduce the number of spots detected in the Western analysis, the 2D-PAGE experiment was repeated using a rehydration buffer (proteins are mixed with this buffer prior to isoelectric focusing) with a higher urea concentration (changed from 8 M to 9.8 M) to promote better protein unfolding because multiple spots could be due to different protein conformations. Despite the change, however, a similar spot pattern as described in Figure 3.1A was observed (Figure 3.1B). There were, however, slight variations in relative spot intensities between the two gels. For example, spot (E) and the minor spot migrating above spot (D) observed in Figure 3.1A are more intense with the increase in urea concentration. However, slight differences in spot intensities are likely not meaningful because the two 2D-PAGE experiments were not performed at the same time, and therefore slight variations in experimental manipulation could lead to slight variations in the results. Overall, the increase in urea concentration did not appear to cause significant changes in protein isoelectric focusing and therefore subsequent 2D-PAGE experiments were performed with rehydration buffer containing 8 M urea as recommended by the 2D-PAGE protocol provided by Amersham Biosciences. Once again, the 2D-PAGE standards could not be detected so meaningful conclusions about the pI values of the detected spots could not be made from the 2D-PAGE results. Two-dimensional PAGE standards were subsequently purchased from Pierce and although these standards were more often detectable by Coomassie staining, they were not detectable in every experiment. Therefore, to eliminate the need of including 2D-PAGE standards, it was decided that 2D-PAGE experiments would be performed to compare protein spot patterns for different cell-free extracts and any spot(s) appearing in one gel but not the other would then be excised for mass spectrometric characterization. The experiments performed with the 30 hour cell-free extract described above were performed once each with either 8 M or 9.8 M urea.

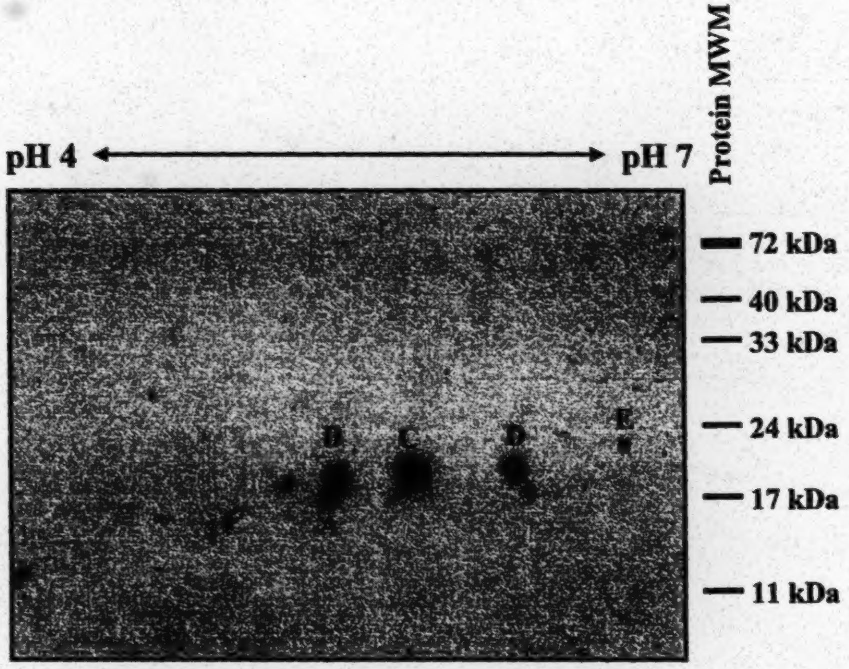
Two-dimensional PAGE experiments were repeated using cell-free extracts (100 μg total protein content) prepared from surface-grown cultures of *S. coelicolor* wild-type strain M600 at 18 hours post-inoculation (corresponds to the transition period between vegetative growth and aerial growth) and 36 hours post-inoculation (corresponds to the transition period between aerial growth and sporulation). In hopes of reducing the number of possible cross-reacting protein spots, Western detection was performed using affinity-purified αBldD antibodies (see Methods section 2.4.6 and Results section 3.1.3). For the 18 hour sample, a similar spot pattern as seen in Figure 3.1 was observed (Figure 3.2A). Although the spot to the left of spot (A) and (B) was as intense as spot (A) and could be considered a major BldD spot (Figure 3.2A), this spot was deemed insignificant because it was not consistently intense in all 2D-PAGE experiments (see again, Figure 3.1A, B and see later, Figure 3.4). The spots in the 36 hour sample were less intense (Figure 3.2B) than the spots in the 18 hour sample but this could be due to the fact that BldD becomes degraded at later time points when grown on solid medium (see later, Figure 3.3A). The spot pattern for the 36 hour sample was somewhat similar to that for the 18 hour sample but, interestingly, the most basic spot, spot (E), seen in the 18 hour sample was significantly less intense in the 36 hour sample (Figure 3.2A, B). As mentioned above, spot (E) may represent unmodified BldD, and therefore the near absence of this spot in the 36 hour sample could reflect the observation that BldD is significantly degraded in the 36 hour surface culture extract (see later, Figure 3.3A). Unfortunately, due to time constraint, the 2D-PAGE experiments described above could not be repeated and therefore it is not known whether the absence of spot (E) in the 36 hour surface culture sample is significant or simply a result of experimental error.

Since the use of affinity-purified αBldD antibodies failed to reduce the number of observed spots to the two expected from the Hesketh study, further attempts were made



(A) 18 hour, surface culture

pH 4
pH 7
Protein MWM
72 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
A
B
D
E

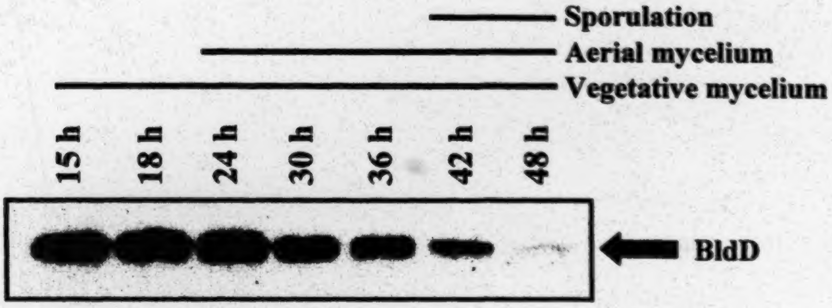


(B) 36 hour, surface culture

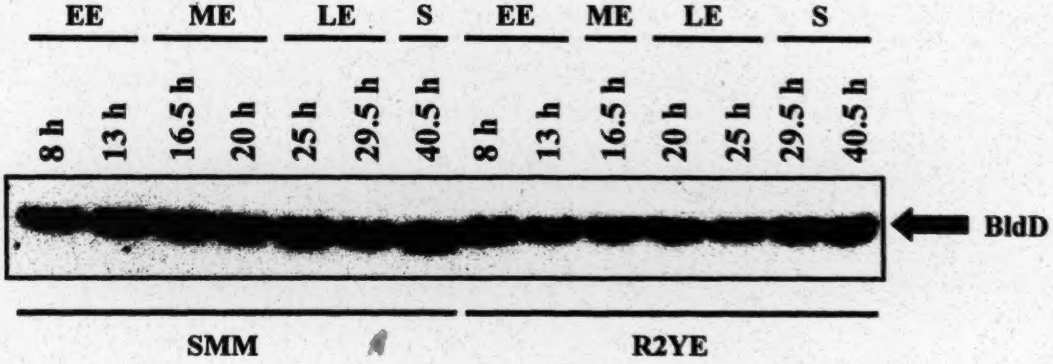
pH 4
pH 7
Protein MWM
72 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
B
C
D
E

to determine which, if any, of the spots could be explained by nonspecific cross-reaction of the antibodies. To this end, 2D-PAGE experiments were again repeated with cell-free extracts prepared from the wild-type M600 strain and a markerless *ΔbldD* null mutant (see later in section 3.1.2 for details of the construction of the *ΔbldD* FLP mutant). At the time of this experiment, three *ΔbldD* mutant strains (1169, *ΔbldD1*, *ΔbldD* FLP 49; see Table 2.1) were available. The 1169 strain was not used because this strain expresses low levels of mutant BldD protein and it was believed that this could lead to confounding 2D-PAGE results. The *ΔbldD1* and *ΔbldD* FLP 49 strains both do not produce any BldD protein and the decision to use the latter strain was entirely arbitrary. In contrast to what is observed when *S. coelicolor* M600 is grown on the surface of agar plates, where BldD protein levels decrease during aerial mycelium formation and sporulation (Figure 3.3A), BldD protein levels are constant at all time points during submerged, liquid growth (Figure 3.3B). Therefore, these 2D-PAGE experiments were performed using cell-free extracts from liquid-grown cultures to determine how the 2D spot pattern for BldD would appear when BldD is not degraded. This result would help address whether the reduction in intensity of spot (E) in the M600 – 36 hour surface culture sample seen in Figure 3.2B was significant. Additionally, it was hoped that by comparing the 2D spot pattern of the wild-type to that of the *ΔbldD* FLP 49 mutant, it would be possible to rule out any spots unrelated to BldD. Two-dimensional PAGE experiments were performed with cell-free extracts (100 μg total protein content) isolated at 8 hours post-inoculation (prior to pigmented antibiotic production = early exponential phase) and 40.5 hours post-inoculation (well after pigmented antibiotic production = stationary phase). The spot patterns for the M600 – 8 hour and M600 – 40.5 hour samples were very similar to each

(A) Surface culture

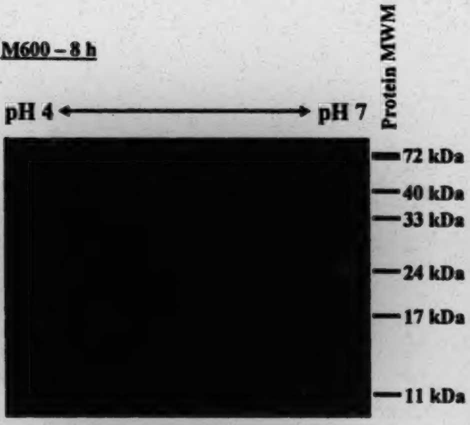

Sporulation
Aerial mycelium
Vegetative mycelium
15 h
18 h
24 h
30 h
36 h
42 h
48 h
BldD

(B) Liquid culture

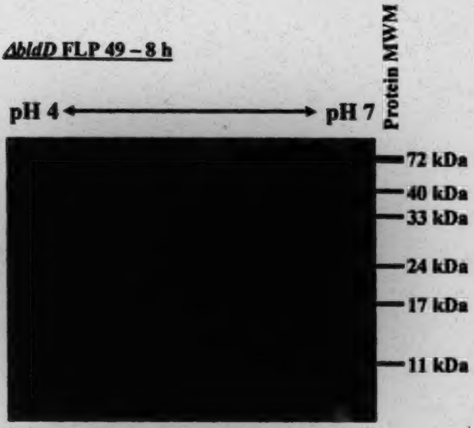

EE
ME
LE
S
EE
ME
LE
S
8 h
13 h
16.5 h
20 h
25 h
29.5 h
40.5 h
8 h
13 h
16.5 h
20 h
25 h
29.5 h
40.5 h
BldD
SMM
R2YE

other (Figure 3.4) and very similar to the pattern seen for the surface-grown cultures (see again, Figure 3.2A, B). It does appear, however, that the reduced intensity of spot (E) seen in the M600 – 36 hour surface culture sample (Figure 3.2B) is similarly observed in the M600 – 40.5 hour liquid culture sample (Figure 3.4). Although this result is interesting, it was initially expected, on the basis of the Hesketh study, that BldD modification would occur at later time points in development so additional spots should theoretically have appeared rather than disappeared in the late time points. For the *ΔbldD* FLP 49 mutant samples, no spots were seen in the 8 hour sample while a single spot was seen in the 40.5 hour sample. Why this single spot appeared only in the 40.5 hour sample and not in the 8 hour sample is unclear. Although this spot could simply be an artifact, no absolute conclusions about the results can be made because this 2D-PAGE experiment was performed only once. Based on the results at hand, it is difficult to rule out any spots seen in the wild-type samples as BldD-unrelated spots because of the seemingly lack of spots in the *ΔbldD* FLP 49 mutant samples. The results appear to suggest that the majority of spots seen in the wild-type samples are related to BldD and are not the result of cross-reaction with αBldD antibodies. Furthermore, the observation of similar BldD spot patterns in cell-free extracts harvested from surface and liquid cultures suggests that the different BldD spots cannot simply be different BldD degradation products. While the multiple BldD spots seen in cell-free extracts harvested from wild-type *S. coelicolor* surface cultures at 36 hours post-inoculation could represent different BldD degradation products because BldD degradation is significant under these growth conditions, the presence of multiple BldD spots in cell-free extracts harvested either from 18 hour surface culture or from liquid culture is not expected because under these growth

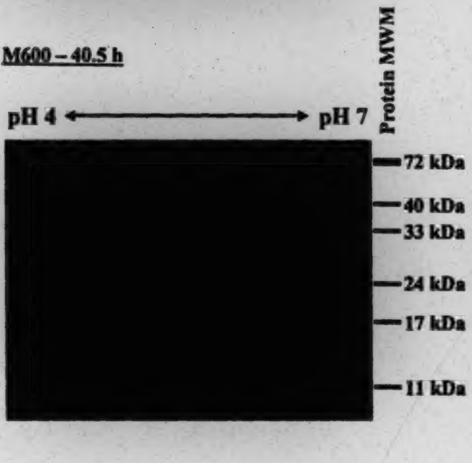
M600 – 8 h


pH 4
pH 7
Protein MWM
72 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa

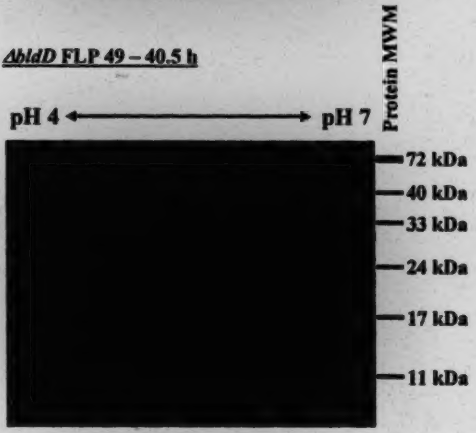
ΔbldD FLP 49 – 8 h


pH 4
pH 7
Protein MWM
72 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa

M600 – 40.5 h


pH 4
pH 7
Protein MWM
72 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa

ΔbldD FLP 49 – 40.5 h


pH 4
pH 7
Protein MWM
72 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa

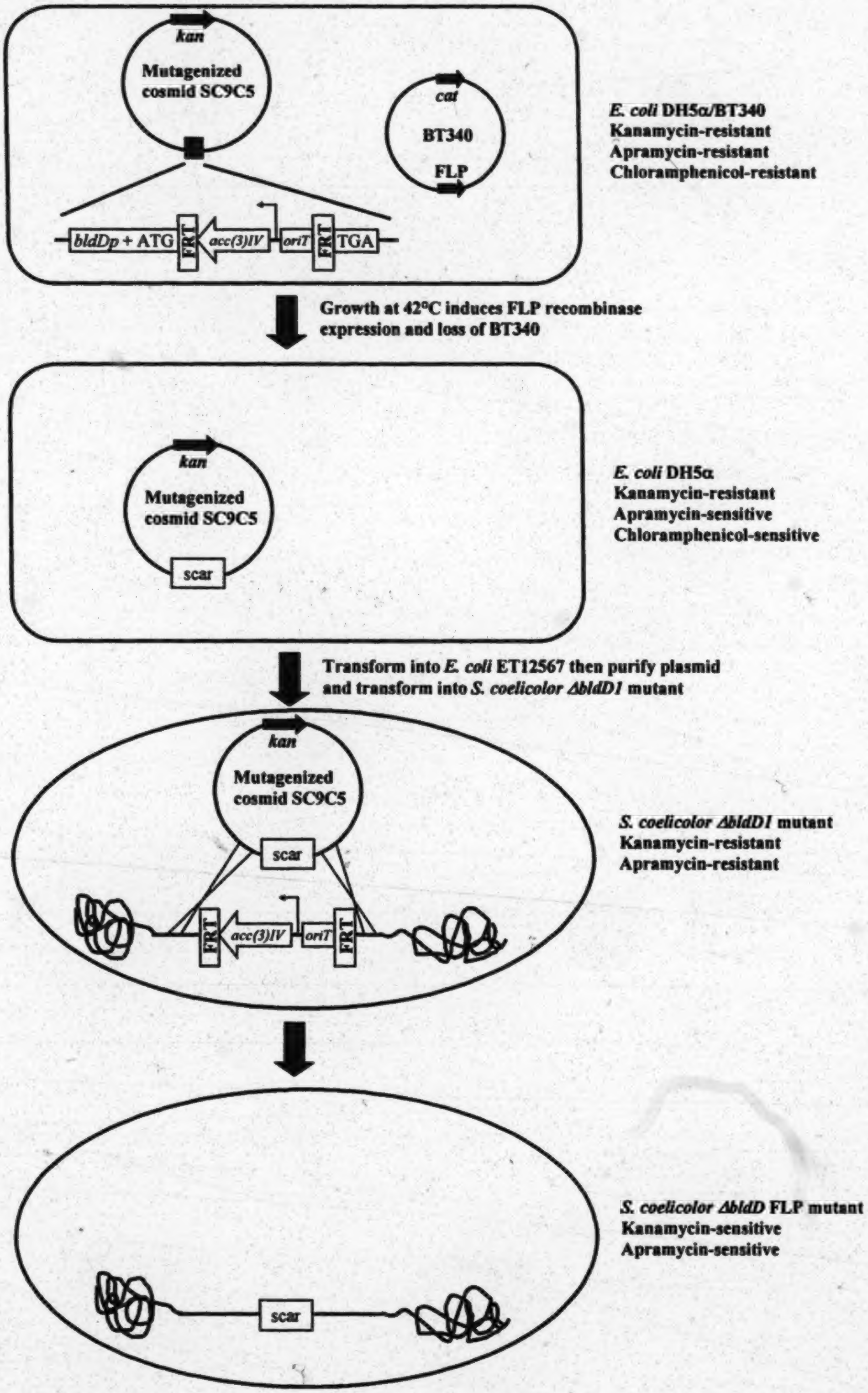
conditions BldD degradation is negligible. However, since the 2D-PAGE experiments described here were only performed once, strong conclusions about the results cannot be made until the results have been confirmed with repeated attempts. The results from this study, although requiring validation experiments, suggest that multiple BldD isoforms may exist. Although A. Hesketh observed only two BldD isoforms, it should be noted that he never performed Western analyses on his 2D gels but rather identified the BldD spots after random spot picking and mass spectrometric analyses of proteins separated by 2D-PAGE. It is therefore not known if additional BldD spots were present on his gels and simply not picked and identified.

In order to determine the nature of the BldD modification, it is critical that protein spots are detectable by Coomassie Brilliant Blue staining so that any spot(s) suspected to be the modified BldD protein can be excised and characterized by mass spectrometry. Therefore, 2D-PAGE experiments were repeated once with cell-free extracts isolated at 8 and 40.5 hours post-inoculation from liquid-grown cultures of the wild-type M600 and *ΔbldD* FLP 49 strains of *S. coelicolor*. To try and increase the chances of detecting spots and obtaining enough protein within spots for mass spectrometric analysis, the total protein content to be resolved was increased from 100 μg to 400 μg. However, due to the increase in total protein content, proper protein focusing was not achieved using the 7 cm isoelectric focusing strips, as indicated by poor first dimension separation of spots (data not shown). Another approach, which has not yet been explored in this thesis, would be to analyze 100 μg total protein content and detect the spots by staining with a more sensitive stain such as Sypro® Ruby (Bio-Rad). Although better spot detection would be achieved, a potential drawback that arises is that there may not be enough protein in the spots to characterize the modification by mass spectrometry. However, this problem may be circumvented if replicate gels of the same protein sample were analyzed and then the protein spots corresponding to unmodified and modified BldD proteins could be combined prior to mass spectrometric analysis.

3.1.2. Large format 2D-PAGE

Since efforts to resolve large amounts of total protein content using 7 cm isoelectric focusing strips and the small format second dimension gels had failed, another approach was pursued involving the use of longer isoelectric focusing strips and larger format second dimension gels. This change was expected to allow not only for better separation of proteins, but also to allow for loading of larger amounts of protein onto the strips. It was hoped that the loading of higher protein content onto the strips would facilitate visualization of protein spots by Coomassie Brilliant Blue staining. For these experiments, the *ΔbldD* FLP 49 mutant harbouring the integrating pIJ6902 expression vector containing *bldD* under the inducible *tipA* promoter (pAU244, Table 2.2) was used to increase the amount of BldD expression. Since the cell-free extract prepared from this strain would be enriched in unmodified BldD protein (due to BldD protein overexpression), and presumably its isoforms, 2D-PAGE experiments would not require great increases in total protein content, therefore facilitating better first dimension separation of proteins. Ultimately, it was hoped that the increase in BldD expression would lead to an increase in the populations of both the unmodified and modified BldD proteins thereby hopefully increasing the probability of detecting the isoforms following Coomassie staining.

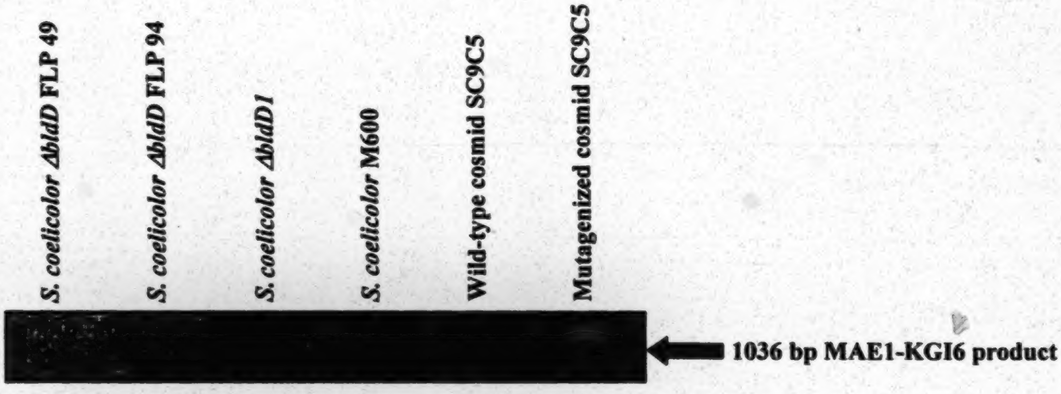
To create a BldD expression system in a *ΔbldD* null background, a markerless *ΔbldD* null strain needed to be constructed because both the existing *ΔbldD1* mutant (Table 2.1) and pAU244 (Table 2.2) carry a marker for apramycin resistance. The *aac(3)IV+oriT* cassette (codes for apramycin resistance), which was used to replace the *bldD* coding region in the *bldD*-containing cosmid SC9C5 (Table 2.2), is flanked by FLP recognition target (FRT) sites so excision of the *aac(3)IV+oriT* cassette would leave behind an in-frame, 81 bp scar sequence. FLP excision of the *aac(3)IV+oriT* cassette followed the REDIRECT© technology (Figure 3.5) (Gust et al. 2003). The mutagenized cosmid SC9C5, which contains the *aac(3)IV+oriT* cassette in place of the *bldD* coding region (Table 2.2; Elliot et al. 2003*b*), was transformed into *E. coli* DH5α harbouring BT340, the temperature-sensitive FLP recombination plasmid. Following growth at 30°C in the presence of 50 μg/mL apramycin (selects for mutagenized cosmid) and 25 μg/mL chloramphenicol (selects for BT340), three random transformants were streaked for single colonies and grown without antibiotic selection at 42°C to induce expression of FLP recombinase and subsequent loss of BT340. Clones that were apramycin-sensitive and kanamycin-resistant, indicating the excision of the *aac(3)IV+oriT* cassette in the mutagenized cosmid SC9C5, were propagated in liquid culture before isolating cosmid DNA for verification of FLP excision by restriction digestion and PCR analysis (data not shown). Cosmid DNA preparations that were verified for FLP excision of the *aac(3)IV+oriT* cassette were then introduced into the non-methylating *E. coli* ET12567 strain prior to introduction into *S. coelicolor* because *S. coelicolor* has a methyl-sensing restriction system. Cosmid DNA purified from *E. coli* ET12567 harbouring the scar-containing cosmid SC9C5 (Table 2.2) was transformed into protoplasts of the *ΔbldD1*


kan
Mutagenized cosmid SC9C5
cat
BT340
FLP
bldDp + ATG
FRT
acc(3)IV
oriT
FRT
TGA
E. coli DH5α/BT340
Kanamycin-resistant
Apramycin-resistant
Chloramphenicol-resistant
Growth at 42°C induces FLP recombinase expression and loss of BT340
kan
Mutagenized cosmid SC9C5
scar
E. coli DH5α
Kanamycin-resistant
Apramycin-sensitive
Chloramphenicol-sensitive
Transform into E. coli ET12567 then purify plasmid and transform into S. coelicolor ΔbldD1 mutant
kan
Mutagenized cosmid SC9C5
scar
FRT
acc(3)IV
oriT
FRT
S. coelicolor ΔbldD1 mutant
Kanamycin-resistant
Apramycin-resistant
scar
S. coelicolor ΔbldD FLP mutant
Kanamycin-sensitive
Apramycin-sensitive

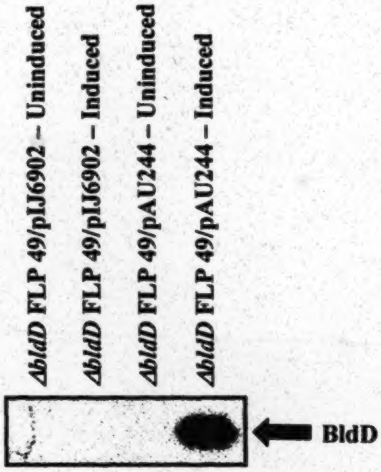
mutant, which contains the chromosomal copy of the *bldD* coding region replaced by the *aac(3)IV+oriT* cassette. A double crossover recombination event between homologous sequences of the scar-containing cosmid SC9C5 and the *ΔbldD1* chromosome would result in excision of the *aac(3)IV+oriT* cassette from the latter to leave behind an in-frame, 81 bp scar sequence. Introduction of the scar-containing cosmid SC9C5 was achieved by protoplast transformation rather than conjugation because excision of the *aac(3)IV+oriT* cassette resulted in the loss of the origin of transfer needed for the process of conjugation. Six kanamycin-resistant primary transformants harbouring the scar-containing cosmid SC9C5 were then grown as patches without antibiotic selection to facilitate isolation of the desired double crossover homologous recombination event in which the region of the *ΔbldD1* chromosome containing the *aac(3)IV+oriT* cassette in place of the *bldD* gene would be replaced with the 81 bp scar sequence. Mycelial stocks (see Methods section 2.1.5) were made of the patches and these were then plated to extinction without antibiotic selection. One hundred of the resulting colonies from the highest dilution plates were then replica-patched first to R2YE then to R2YE supplemented with apramycin and nine patches were found to be apramycin-sensitive. These nine patches were replica-patched first to R2YE then to R2YE supplemented with kanamycin and three patches were observed to be sensitive to apramycin and kanamycin. PCR analysis using the primers MAE1 (flanks upstream sequence of the *bldD* coding region) and KGI6 (internal to *aac(3)IV+oriT* cassette) along with isolated genomic DNA from two of these FLP strains (49 and 94) as template, indicated loss of the *aac(3)IV+oriT* cassette in both of the strains (Figure 3.6A).

Introduction of pAU244 and the *attB*-site integrating parent plasmid, pIJ6902

(A) Confirmation of FLP excision


S. coelicolor ΔbldD FLP 49
S. coelicolor ΔbldD FLP 94
S. coelicolor ΔbldD1
S. coelicolor M600
Wild-type cosmid SC9C5
Mutagenized cosmid SC9C5
1036 bp MAE1-KGI6 product

(B) Confirmation of inducible BldD expression


ΔbldD FLP 49/pIJ6902 – Uninduced
ΔbldD FLP 49/pIJ6902 – Induced
ΔbldD FLP 49/pAU244 – Uninduced
ΔbldD FLP 49/pAU244 – Induced
BldD

(Huang et al. 2005), into the *ΔbldD* FLP 49 mutant was mediated by conjugation (see Methods section 2.2.6). Conjugation from *E. coli* to *Streptomyces* was made possible because both pIJ6902 and its pAU244 derivative contain *oriT*, an origin of transfer obtained from a broad host range plasmid. BldD expression, as monitored by Western analysis of cell-free extract isolated from this strain following thiostrepton induction, was performed to confirm the inducible expression of BldD from the integrated plasmid. BldD expression was observed in the *ΔbldD* FLP 49/pAU244 strain following three hour thiostrepton induction while no BldD expression was observed for the uninduced control of the same strain (Figure 3.6B). As expected, no BldD expression was observed for the strain harbouring the parent pIJ6902 plasmid under both uninduced and induced conditions.

Following verification of proper construction of the *ΔbldD* FLP 49/pAU244 strain, cell-free extract was harvested from this strain after three hours induction with thiostrepton. Total cellular protein (900 μg) was mixed with rehydration buffer and used to swell a long Immobiline® DryStrip gel (18 cm, pH 4-7) while a second strip was used to focus 2D-PAGE standards (Pierce). Although it was promising that many spots could be detected after Coomassie staining of the 2D-PAG, the 2D-PAGE standards could not be detected so it was not possible to use pI value, together with molecular weight, to estimate the location of the BldD protein (data not shown). This approach was abandoned after only one attempt because it was believed that too much optimization would be required to obtain gels with discernable BldD spots. Due to the lack of success in isolating different BldD forms by 2D-PAGE, it was decided that an immuno-affinity technique would instead be employed to isolate the BldD isoforms.

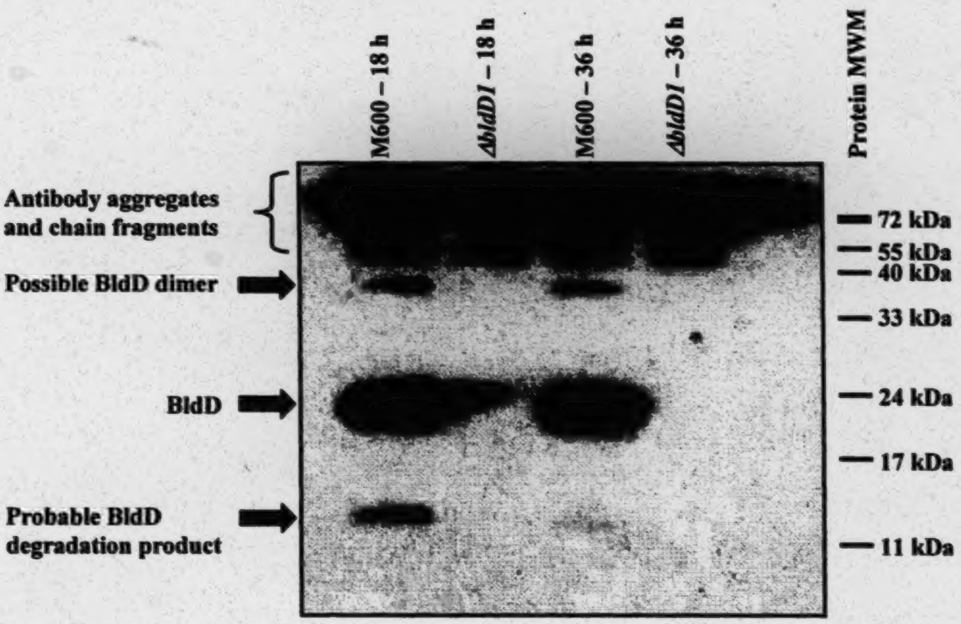
3.1.3. Immunoprecipitation (IP)

An immunoprecipitation technique was employed in an attempt to isolate the BldD isoforms from cell-free extracts of *S. coelicolor* with the eventual goal to characterize these protein forms by mass spectrometry. The IP technique is a powerful technique for the isolation and concentration of proteins expressed at low levels. This sensitive technique was expected to facilitate effective isolation of the modified BldD proteins, which are likely less abundant than unmodified BldD protein. The idea was to isolate the BldD isoforms from *S. coelicolor* cultures at different time points during development with the hope that extracts from later time points would be enriched for the modified BldD protein that had been previously observed by A. Hesketh. To ensure that antibodies would be specific for the purification of BldD protein alone, αBldD antibodies were affinity-purified from the crude antibody preparation (see Methods section 2.4.6). The affinity purification was accomplished by precipitating IgG from the crude antibody preparation using ammonium sulfate (40% saturation) followed by binding the ammonium sulfate-precipitated IgG to purified His$_6$-BldD protein coupled to CNBr-activated Sepharose 4B. The antibody was then released from the His$_6$-BldD protein by washing with glycine (pH 2.0) followed by immediate neutralization with Tris-HCl (pH 8.0), and subsequent dialysis against PBS buffer. It was previously shown that antibodies purified in this manner did not cross-react with any proteins in cell-free extract harvested from a *ΔbldD* null mutant (see again, Figure 3.4).

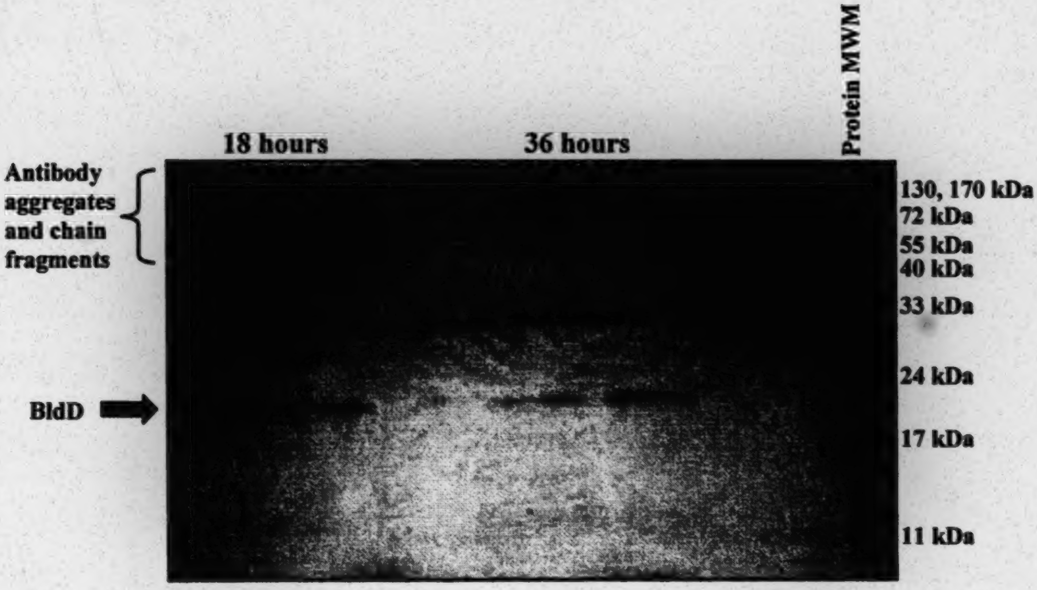
The immunoprecipitation of BldD involved first mixing cell-free extracts with the affinity-purified αBldD antibodies to allow complex formation between BldD and the αBldD antibodies. The resulting immunocomplexes were then captured with protein A Sepharose and the immunocomplexes were eluted/dissociated from the protein A Sepharose beads by boiling in non-reducing SDS-PAGE buffer. Non-reducing SDS-PAGE buffer was used to maintain disulfide bonds within the antibody heavy and light chains thereby minimizing contamination of the BldD bands with antibody chain fragments. The beads were removed by centrifugation and the supernatant containing the eluted immunocomplexes were subsequently analyzed by SDS-PAGE. For this study, IP reactions were performed using cell-free extracts from surface-grown cultures of *S. coelicolor* wild-type strain M600 harvested at 18 hours post-inoculation (corresponds to the transition period between vegetative growth and aerial growth) and 36 hours post-inoculation (corresponds to the transition period between aerial growth and sporulation). As a negative control, IP reactions were also performed with cell-free extracts harvested from the *S. coelicolor ΔbldD1* strain at the same time points post-inoculation.

Using this methodology, BldD protein was detected in M600 cell-free extracts by Western analysis (with little detectable contamination other than antibody aggregates/fragments and slight BldD degradation) and this band was absent from IP samples using extracts isolated from the *ΔbldD1* mutant (Figure 3.7A). For mass spectrometric analysis of immunoprecipitated BldD, the IP experiment was repeated doing three reactions each with 18 hour and 36 hour cell-free extracts of the M600 strain. The eluted IP sample for each extract was pooled and analyzed by SDS-PAGE followed by Coomassie staining of the gel (Figure 3.7B). The bands corresponding to the apparent molecular weight of BldD protein were then excised from the gel and characterized by mass spectrometry at the Institute for Biomolecular Design (University of Alberta). To

(A) Western detection of immunoprecipitated BldD


M600 – 18 h
ΔbldD1 – 18 h
M600 – 36 h
ΔbldD1 – 36 h
Protein MWM
Antibody aggregates and chain fragments
Possible BldD dimer
BldD
Probable BldD degradation product
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa

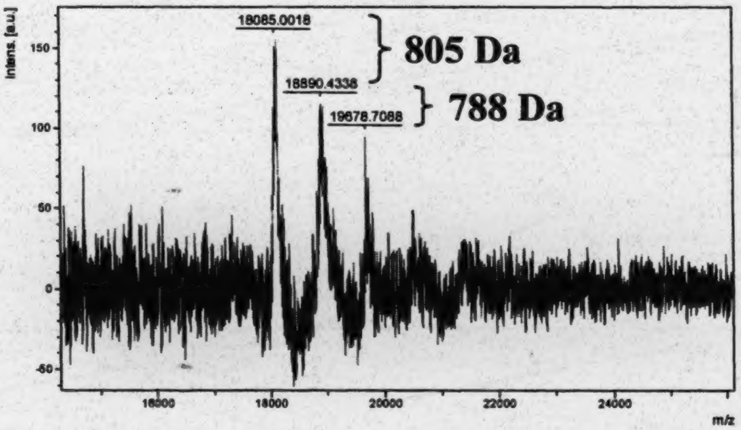
(B) Coomassie staining of immunoprecipitated BldD


18 hours
36 hours
Protein MWM
Antibody aggregates and chain fragments
BldD
130, 170 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa

obtain clues about the presence of other BldD isoforms in the IP samples, intact total molecular weights of protein species in the gel were determined. In-gel tryptic digests were also performed on the protein in the gel slices and the resulting fragments were subjected to liquid chromatography tandem mass spectrometry (LC MS/MS) to identify the protein. LC MS/MS and database searches of the tryptic digests confirmed that the protein isolated by immunoprecipitation from *S. coelicolor* M600 at 18 and 36 hours was the BldD protein. The peaks observed in the total molecular weight determination analyses for the 18 and 36 hour IP samples were essentially the same except that the peak intensities for the 36 hour sample were lower (Figure 3.8), most likely due to the lower amount of immunoprecipitated BldD in this sample (Figure 3.7B). Initially it was promising that there were two larger peaks in addition to the peak matching most closely to the predicted molecular weight of BldD without the N-formyl-methionine (18 047 Da). Each peak differed in mass-to-charge ratio (m/z) by ~800 Da (recall that A. Hesketh observed a BldD isoform ~1 kDa larger than unmodified BldD) so it was thought that there may be two similar modifications happening sequentially on BldD. It was later discovered, however, that the additional peaks were likely just Coomassie Brilliant Blue stain adducts. In conclusion, although this methodology confirmed that the immunoprecipitated protein was BldD, it did not lead to any information about any putative BldD modifications. From this finding, it was assumed that the population of modified BldD proteins, if they exist, was either too low or too unstable in the immunoprecipitated protein sample to allow for detection by mass spectrometry.

To try and increase the amount of BldD protein isolated, the next approach pursued involved pooling the immunoprecipitated proteins from 12 IP reactions and

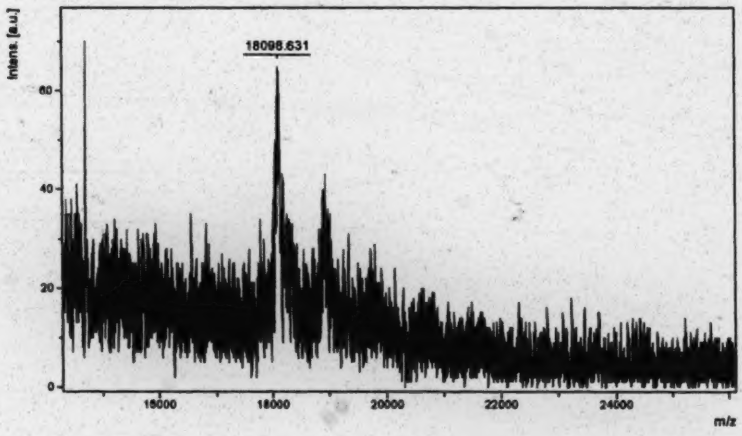
18 hours



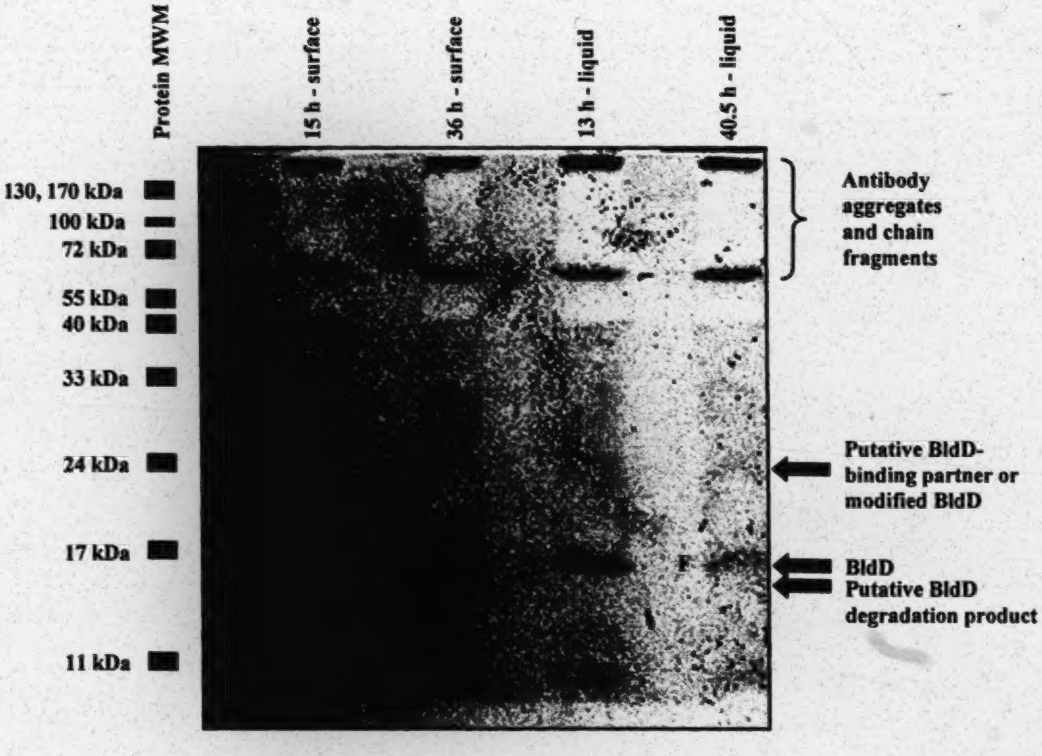
805 Da

788 Da

36 hours



separating the IP samples on a large SDS-PAG. It was hoped that within the larger IP sample, there would be enough modified BldD protein for characterization by mass spectrometry. IP reactions were performed using cell-free extracts of surface-grown cultures of *S. coelicolor* M600 harvested at 15 hours post-inoculation (corresponds to vegetative growth) and 36 hours post-inoculation (corresponds to the transition period between aerial growth and sporulation). IP reactions were also performed with cell-free extracts of liquid-grown (R2YE) cultures of wild-type M600 harvested at 13 hours post-inoculation (early-exponential phase) and 40.5 hours post-inoculation (stationary phase). Following SDS-PAGE of the IP samples and Coomassie staining of the gel, six distinct bands (labeled A-F) could be observed (Figure 3.9). Since protein samples were electrophoresed on a 12% SDS-PAG rather than the usual 15% SDS-PAG, BldD protein was expected to migrate at an apparent molecular weight more closely matching its theoretical molecular weight of 18.2 kDa. Bands (A), (C), (E), and (F) were expected to contain a mixture of unmodified and modified BldD protein. Recall that Western analysis of wild-type *S. coelicolor* cell-free extracts subjected to two-dimensional separation had detected three BldD isoforms, spots (B), (C), and (D), which have similar molecular weight sizes (see again, Figure 3.1, 3.2, and 3.4). The most basic BldD isoform, spot (E), which was observably larger in molecular weight size than the other three isoforms following two-dimensional separation, was expected to migrate apart from the other three species following one dimensional separation. However, examination of Figure 3.9 does not reveal a slightly higher molecular weight band corresponding to spot (E) in any of the IP samples. Interestingly, in the 36 hour surface culture IP sample, there were two additional bands that were absent from the other samples, a slightly smaller


Protein MWM
15 h - surface
36 h - surface
13 h - liquid
40.5 h - liquid
130, 170 kDa
100 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
Antibody aggregates and chain fragments
Putative BldD-binding partner or modified BldD
BldD
Putative BldD degradation product

band likely to be a BldD degradation product, and a band migrating near the 24 kDa marker. This latter band was believed to represent either a BldD-binding partner or one of the modified BldD isoforms. Since BldD belongs to the same family of transcriptional regulators as SinR, a *B. subtilis* repressor protein whose activity is abolished when bound to SinI (Bai et al. 1993), it is plausible to hypothesize that BldD too may have a binding partner to help regulate its function. If the binding affinity between BldD and its binding partner is strong enough to withstand the sonication used to lyse cells for preparation of the cell-free extracts used in the IP reactions, the immunoprecipitation of BldD would also result in the isolation of the BldD-binding partner. All six bands (A-F) were excised for mass spectrometric characterization. Tryptic digestion was performed on the six bands and the resulting peptide mass fingerprints for bands (A), (C), (D), (E), and (F) were compared. Peptide mass fingerprint analysis, rather than LC MS/MS, was performed because the former technique allows for a facile comparison between peptide species in different protein samples. Any peptide(s) present in one sample but not another could then be examined closer to hopefully gain information about the modification(s). Bands (A), (C), (D), (E), and (F) all contained peptides matching unmodified BldD protein (data not shown). However, the peptide mass fingerprints did not reveal any new information about any putative BldD modification(s). Although there were differences in the peptide mass fingerprints for the different protein bands, none appeared to be interesting (data not shown). The reason being that small differences in peptide masses are likely due to modifications occurring on the fragments during the analysis. Also, since the peak intensity threshold was set at the same level for all IP samples, depending on the amount of contaminating material in each IP sample, slight differences in the number of peaks would be observed. In other words, if protein sample A and B are identical except that sample A contains more contaminating material than sample B, then sample A, in addition to containing the same peaks as sample B, would also contain additional peaks representing contaminating material. LC MS/MS was performed on the tryptic digest for band (B) and subsequent database searches were done to find a possible identity for this higher molecular weight protein. Again, the result was disappointing, as the protein in band (B) was found to be a match for albumin. For whatever reason, this IP sample retained a lot of contaminating albumin used in the IMP buffer to block non-specific protein binding. The results described for the large scale IP are based on a single experiment.

From these results it was concluded that the immunoprecipitation technique alone was not sufficient to obtain information about the BldD modification(s). Without separating the protein forms, the small amount of modified BldD proteins would inevitably be masked by the presumably larger proportion of unmodified BldD protein. Furthermore, additional care needed to be taken to separate the BldD protein forms because peptide mass fingerprint data are only useful when protein samples are pure.

3.1.4. Combined IP and 2D-PAGE

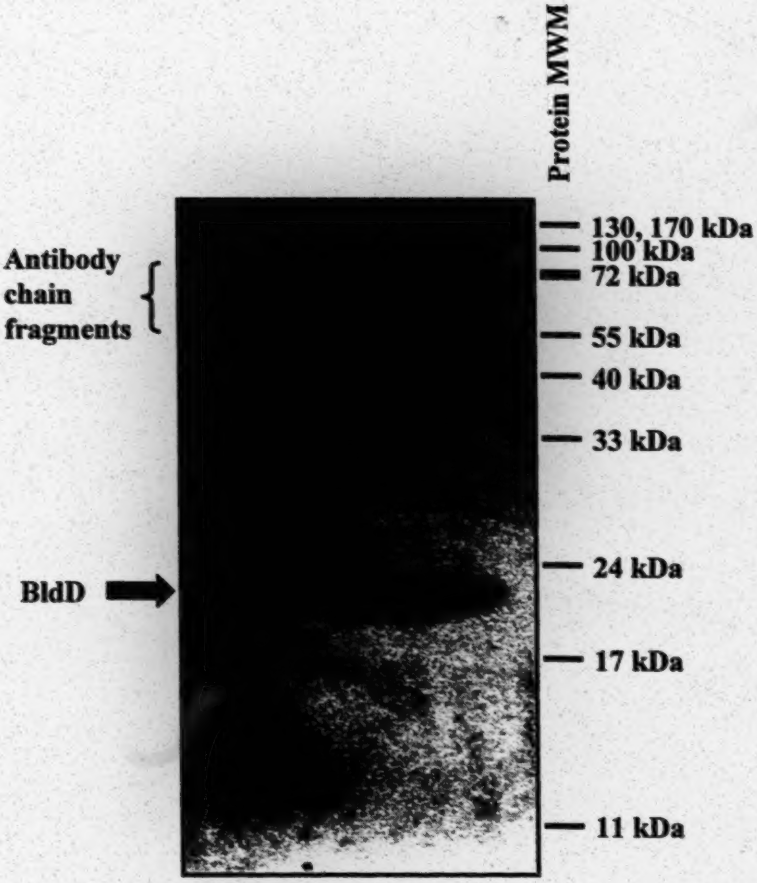
As stated above, previous attempts to isolate and identify modified BldD using 2D-PAGE separation or immunoprecipitation had failed. It was therefore decided that a combination of the IP and 2D-PAGE techniques may lead to information about the BldD modification(s). The experimental plan was to subject immunoprecipitated BldD protein to 2D-PAGE to separate the BldD isoforms before characterization by mass spectrometry. Based on the Hesketh 2D-PAGE study, it was expected that BldD modification would occur at later time points during development, so the decision was made to perform IP reactions with cell-free extract from surface-grown *S. coelicolor* M600 harvested at 36 hours post-inoculation (corresponds to the transition period between aerial growth and sporulation). Three replicate IP reactions were performed and the immunocomplexes were captured with protein A Sepharose as previously described (see Methods section 2.4.8 and Results section 3.1.3). Although the immunocomplexes would normally be dissociated from the protein A Sepharose beads by boiling the immunocomplexes/beads mixture in non-reducing SDS-PAGE buffer, this step in the IP procedure needed to be modified because the PAGE buffer contains SDS, a charged detergent that is incompatible with isoelectric focusing. The immunocomplexes/beads mixture was instead boiled in a 2% solution (w/v) of CHAPS, a zwitterionic detergent that is compatible with isoelectric focusing. Following centrifugation to remove the protein A Sepharose beads, the eluted IP samples from the three replicate IP reactions were pooled, mixed with rehydration buffer, and then used to swell a 7 cm Immobiline® DryStrip gel. Following isoelectric focusing and second dimension separation on a mini 15% SDS-PAG, protein spots were detected by staining with Coomassie Brilliant Blue. This experiment yielded no spots on the gel (data not shown).

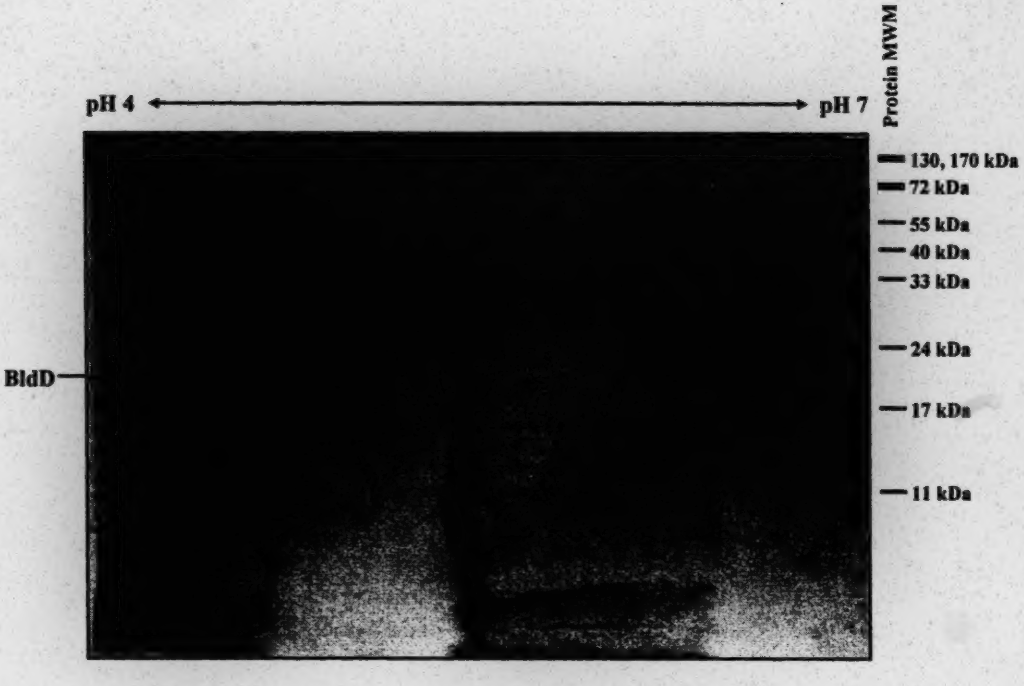
Since it was concluded that too little BldD protein was present in three IP samples, a decision was made to increase the amount of total protein loaded onto the isoelectric focusing strips by performing 10 replicate IP reactions and pooling the eluted IP samples before analyzing by 2D-PAGE. Before doing so, however, several potential problems needed to be addressed. First, pooling the eluted IP samples from 10 IP reactions would give a volume of at least 100 μL. If this large volume was mixed with rehydration buffer there would be a significant dilution of the rehydration buffer components, which would then affect proper isoelectric focusing of protein samples. Although this problem could be circumvented by making a more concentrated rehydration buffer, this was not possible because the urea present in the buffer would not dissolve at a concentration greater than 9.8 M. However, the urea concentration would need to be much higher than 9.8 M because proper isoelectric focusing requires a final urea concentration of at least 8 M. Therefore, it was decided that the volume of the pooled IP sample would be reduced by concentrating the sample by vacuum centrifugation. However, due to the possibility of difficulty in redissolving the concentrated CHAPS precipitate from 10 IP reactions, it was decided to use mQH_2O instead of CHAPS solution to elute the immunocomplexes from the protein A Sepharose beads. Second, since it was possible that the initial combined IP and 2D-PAGE experiment had not worked because too much BldD degradation had occurred in the 36 hour surface culture extract, it was decided to perform IP reactions with cell-free extract harvested from surface-grown M600 at 18 hours post-inoculation to hopefully recover more intact BldD protein.

A trial large scale IP experiment was performed to test the new changes in the IP procedure and to ensure that enough immunoprecipitated protein would be recovered from 10 IP reactions for 2D-PAGE analysis. The immunocomplexes from 10 IP reactions were captured with protein A Sepharose beads and the immunocomplexes were recovered by boiling the protein A Sepharose beads in mQH_2O. The eluted IP samples from all 10 IP reactions were pooled, the pooled sample concentrated, mixed with non-reducing SDS-PAGE buffer, boiled briefly, and then analyzed by SDS-PAGE on a mini

15% SDS-PAG. As can be seen in Figure 3.10, a large band corresponding to the size of BldD was observed following Coomassie staining of the gel. Since boiling in mQH_2O released enough intact BldD protein from the antibodies bound to the protein A Sepharose beads, the large scale IP experiment was therefore repeated and followed by 2D-PAGE. Using this method, however, it was found that although the amount of BldD protein loaded onto the isoelectric focusing strip was indeed increased, so too was the amount of antibody released from the protein A Sepharose beads. It was surprising that there was so much antibody contamination of the IP sample since only a small amount of antibody chain fragments could be seen in Figure 3.10. However, the absence of the higher molecular weight bands corresponding to antibody aggregates is strange and the reason for this absence is unknown. At any rate, the combination of the IP and 2D-PAGE techniques again did not lead to successful separation of the BldD isoforms. After the first dimension electrophoresis, there was a visible bulge roughly in the center of the isoelectric focusing strip (a symptom of protein overloading) and observation of the gel after second dimension separation showed a single BldD spot with significant vertical streaking, suggesting that proper isoelectric focusing had not occurred (Figure 3.11).

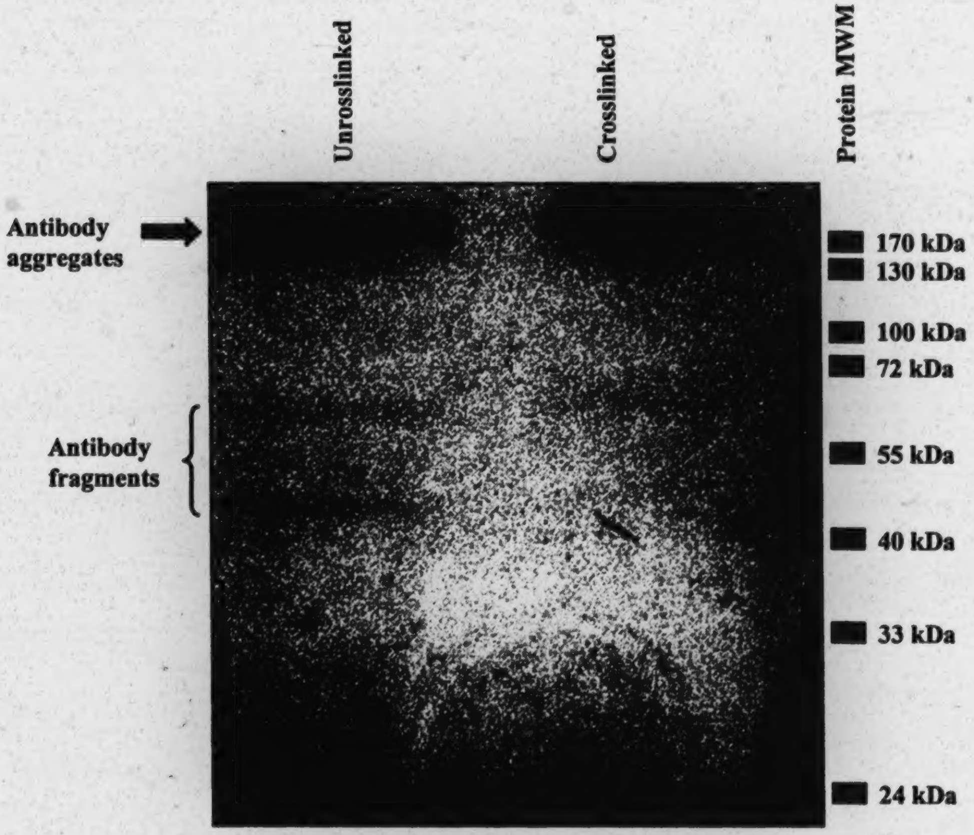
To try to reduce the amount of antibody fragment contamination of the eluted protein samples, a decision was made to irreversibly couple the αBldD antibodies to the protein A Sepharose resin using the chemical crosslinker, DMP (see Methods section 2.4.7). The crosslinking reaction was expected to stabilize the interaction between the αBldD antibodies and the protein A Sepharose resin by facilitating covalent attachments between the antibody and protein A. This antibody-coupled resin would then be used in the IP reaction and it was expected that the boiling of the immunocomplexes/beads


Protein MWM
130, 170 kDa
100 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
Antibody chain fragments
BldD


pH 4
pH 7
Protein MWM
130, 170 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
BldD

mixture would result in the elution of BldD protein from the beads while the αBldD antibodies would remain attached to the beads. Before using the antibody-coupled resin in the IP reaction, SDS-PAGE was performed to compare antibody-coupled resin samples before and after the crosslinking reaction. From Figure 3.12, it appears that the chemical crosslinking had not effectively stabilized the interaction between the antibodies and protein A Sepharose beads because there was significant antibody contamination in both the uncrosslinked and crosslinked resin after boiling. Nevertheless, the combined IP and 2D-PAGE experiment was repeated once again using the coupled-antibody preparation. Not surprisingly, the IP sample did not focus properly and this was likely due to too much antibody contamination. This attempt yielded the same results as seen in Figure 3.11 (data not shown).

In conclusion, the combination of IP and 2D-PAGE techniques did not lead to characterization of the BldD modification(s). Attempts to optimize the crosslinking of antibodies to protein A Sepharose beads may help to overcome the antibody contamination problem that had prevented this experimental procedure from working. To increase the amount of immunoprecipitated BldD protein for 2D-PAGE analysis, cell-free extract from surface-grown *S. coelicolor* M600 harvested at 18 hours post-inoculation were used because IP experiments were found to be more successful with extracts isolated from early time points in growth. If the antibody contamination problem could have been overcome, it would then have been necessary to use cell-free extracts from an entire time course because the 2D-PAGE results in this study suggest modification may occur early in growth while the Hesketh results suggest the opposite.

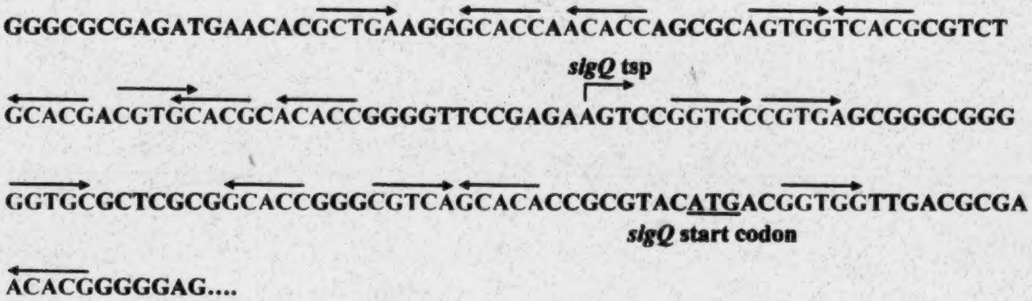

Unrosslinked
Crosslinked
Protein MWM
Antibody aggregates
Antibody fragments
170 kDa
130 kDa
100 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa

3.1.5. Use of promoter fragments as bait to capture BldD isoforms

Due to the lack of success in isolating the BldD isoforms using the 2D-PAGE and IP techniques, a DNA affinity chromatography technique was pursued. This technique involves the use of target DNA as bait to capture a DNA-binding protein of interest. Previously Kelemen et al. (2001) were able to isolate BldD from cell-free extract of wild-type *S. coelicolor* by using a DNA fragment that contained the *sigH* promoter sequence. Previous microarray analyses of global gene expression following *bldD* induction revealed that in addition to repressing several target genes as expected, BldD appears to activate the expression of *sigQ* (C. Galibois, unpublished). Based on this finding, it was hypothesized that the repression and activation of target gene expression by BldD may involve different forms of BldD. Since BldD is known to repress transcription of its own gene and is predicted to activate transcription of *sigQ*, it was further hypothesized that BldD would bind to the *bldD* promoter fragment in its repressor, likely unmodified, form and that it might bind to the *sigQ* promoter fragment in its activator, perhaps modified, form. Purification of BldD protein bound to each of the target DNA fragments and subsequent characterization by mass spectrometry might then reveal differences between the bound proteins. Alternatively, if the switch between the BldD repressor and activator functions is dependent on the interaction with another protein, this technique might also lead to the identification of a bound protein partner(s).

The approach to immobilize the promoter fragments onto a column for isolation of BldD protein involved using the μMACS streptavidin kit purchased from Miltenyi Biotec. This system involves mixing biotinylated target DNA with cell-free extract containing the DNA-binding protein of interest. Following incubation to allow binding of the protein of interest to the target DNA, streptavidin-coated magnetic microbeads are then added to capture the protein-DNA complexes. The streptavidin beads with the attached protein-DNA complexes are immobilized on a metal column when placed in the magnetic field of a µMACS separator. The column is then washed to remove any nonspecifically bound protein and then the protein bound to the target DNA is eluted with the appropriate elution buffer. For this study, biotinylation of target DNA was accomplished by PCR amplification. For each primer pair used for target DNA amplification, one primer was synthesized with the addition of one biotin unit on the 5' end of the sequence. To minimize nonspecific binding by proteins other than BldD, the size of the target DNA fragments was kept as small as possible by amplifying fragments that included the consensus sequence for BldD binding with little flanking sequence. Promoter fragments previously confirmed to be bound by BldD were chosen for this study (Elliot and Leskiw 1999; C. Galibois, unpublished). To amplify the *bldD* promoter region, the primers LBU2 and MAE4 were used to amplify a fragment of 106 bp. The primer LBU1 used with either the primers CGA41 or CGA52 was used to amplify, respectively, a fragment of 170 bp containing the entire *sigQ* promoter region or a fragment of 83 bp containing the upstream half of the *sigQ* promoter region. The *sigQ* promoter region contains several potential BldD binding sites (Figure 3.13A). While the *bldD* promoter region contains a single BldD binding site consisting of an imperfect inverted repeat sequence (Elliot et al. 2001), the *sigQ* promoter region contains many repeat sequences that resemble half sites of the BldD binding consensus, and the arrangement of half sites is not clearly defined (C. Galibois, personal communication). The 170 bp fragment contains 15 repeat sequences while the 83 bp fragment contains 9

(A) Putative BldD binding sites in *sigQ* promoter region



GGGCGCGAGATGAACACGCTGAAGGGCACCAACACCAGCGCAGTGGTCACGCGTCT

sigQ tsp

GCACGACGTGCACGCACACCGGGGTTCCGAGAAGTCCGGTGCCGTGAGCGGGCGGG

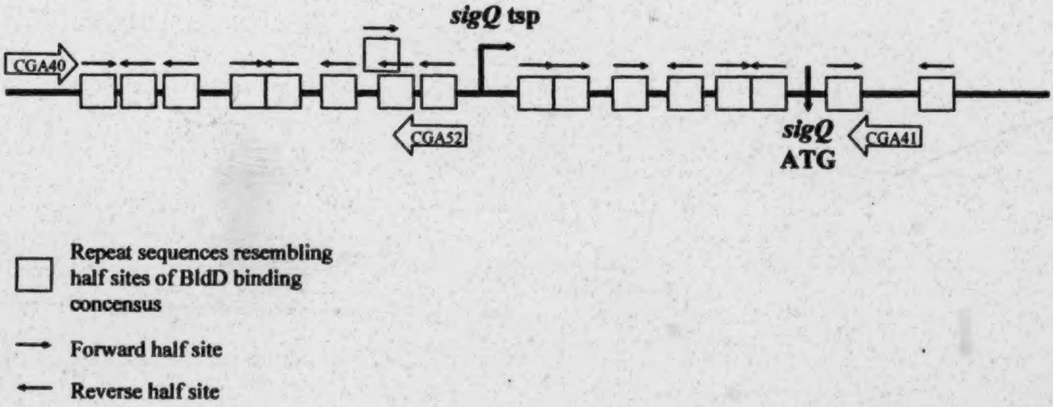
GGTGCGCTCGCGGCACCGGGCGTCAGCACACCGCGTACATGACGGTGGTTGACGCGA

sigQ start codon

ACACGGGGGAG....

BldD binding consensus: a/gGTGa/g/c (n_m) g/tCACg/c

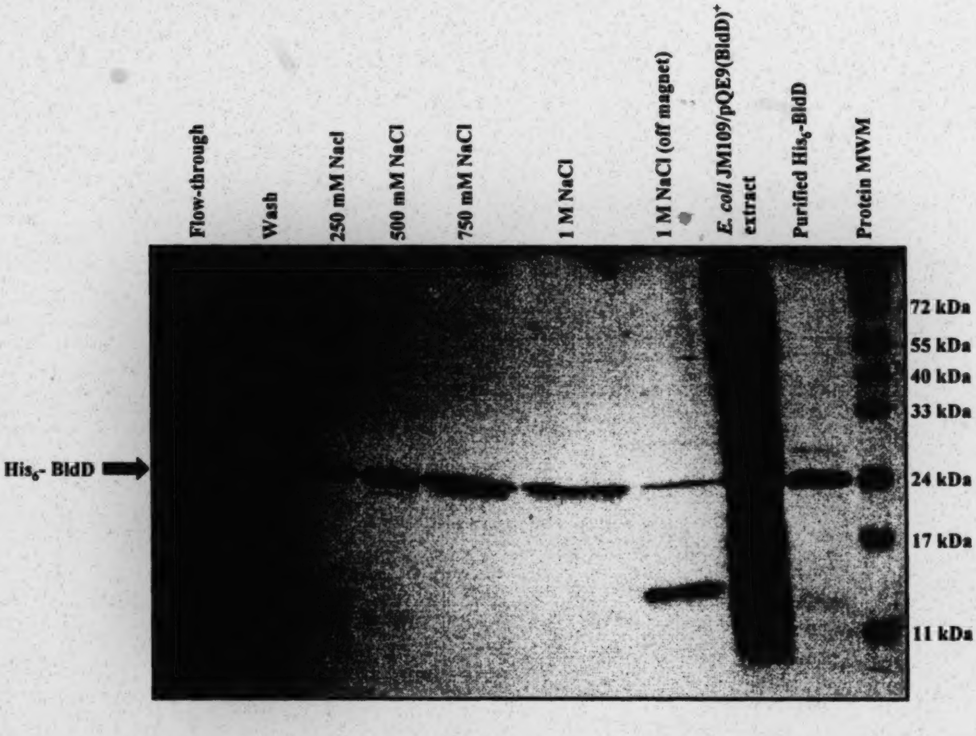
(B) Schematic representation of BldD binding sites in *sigQ* promoter region


sigQ tsp
CGA40
CGA52
sigQ
ATG
CGA41
Repeat sequences resembling half sites of BldD binding concensus
Forward half site
Reverse half site

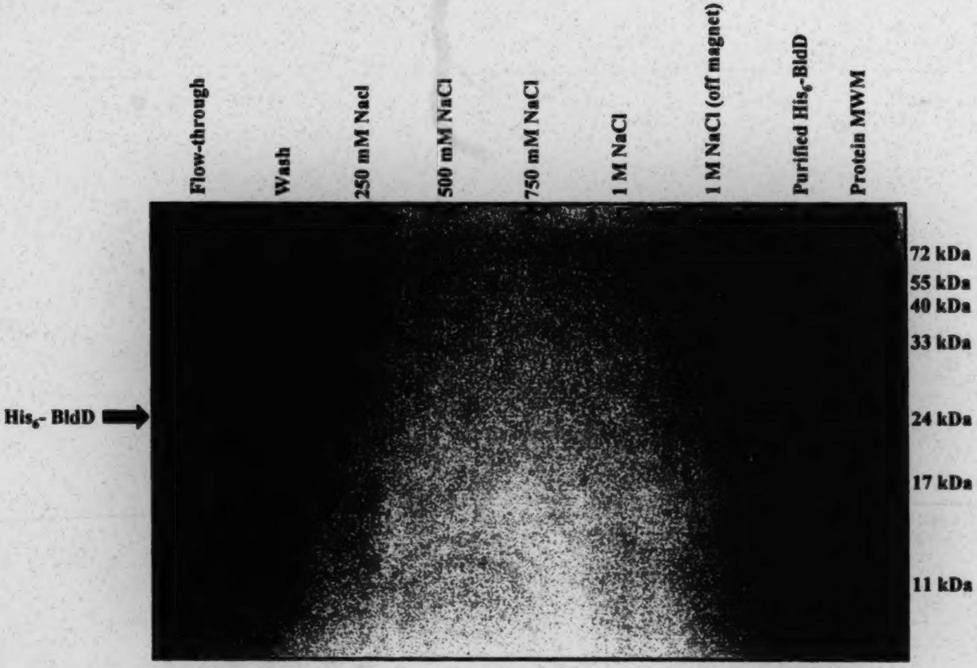
repeat sequences (Figure 3.13B).

Control experiments to test binding conditions were first done using cell-free extracts purified from *E. coli* JM109/pQE(BldD)$^+$ overexpressing the His$_6$-BldD previously used in DNA-binding assays (Elliot and Leskiw 1999; C. Galibois, unpublished). Cell-free extract containing 2.5-3 mg total protein content was mixed with 6-10 μg biotinylated target DNA and incubated to allow for binding of His$_6$-BldD to the target DNA fragment. After addition of the streptavidin-coated magnetic beads and brief incubation, the binding reaction was applied onto the μMACS column, the flow-through collected, and the column washed. BldD protein was then eluted using an NaCl step gradient from 0.25 M to 1 M. The fractions (except for the flow-through) were concentrated and were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Using the *bldD* promoter fragment as bait, a protein band corresponding to the size of the His$_6$-BldD protein was isolated (Figure 3.14A). The purity and abundance of the His$_6$-BldD protein was greatest in the wash fraction containing 750 mM NaCl. An additional lower molecular weight band, migrating between the 11 and 17 kDa molecular weight marker bands, was also observed in the wash fraction where the μMACS column was removed from the magnetic separator. The identity of this band is unknown, although it is likely that this band is somehow related to the streptavidin microbeads because this wash fraction contains the eluted beads. This experiment was performed twice with similar results. Western analysis was not performed to confirm that the purified protein band was in fact His$_6$-BldD protein. In contrast to the results with the *bldD* promoter fragment, His$_6$-BldD protein could not be isolated using either of the *sigQ* promoter fragments after a total of four attempts (Figure 3.14B; only the results for the

(A) *bldD* promoter as bait to capture His$_6$-BldD


Flow-through
Wash
250 mM Nacl
500 mM NaCl
750 mM NaCl
1 M NaCl
1 M NaCl (off magnet)
E. coli JM109/pQE9(BldD)† extract
Purified His6-BldD
Protein MWM
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
His6- BldD

(B) *sigQ* promoter as bait to capture His$_6$-BldD

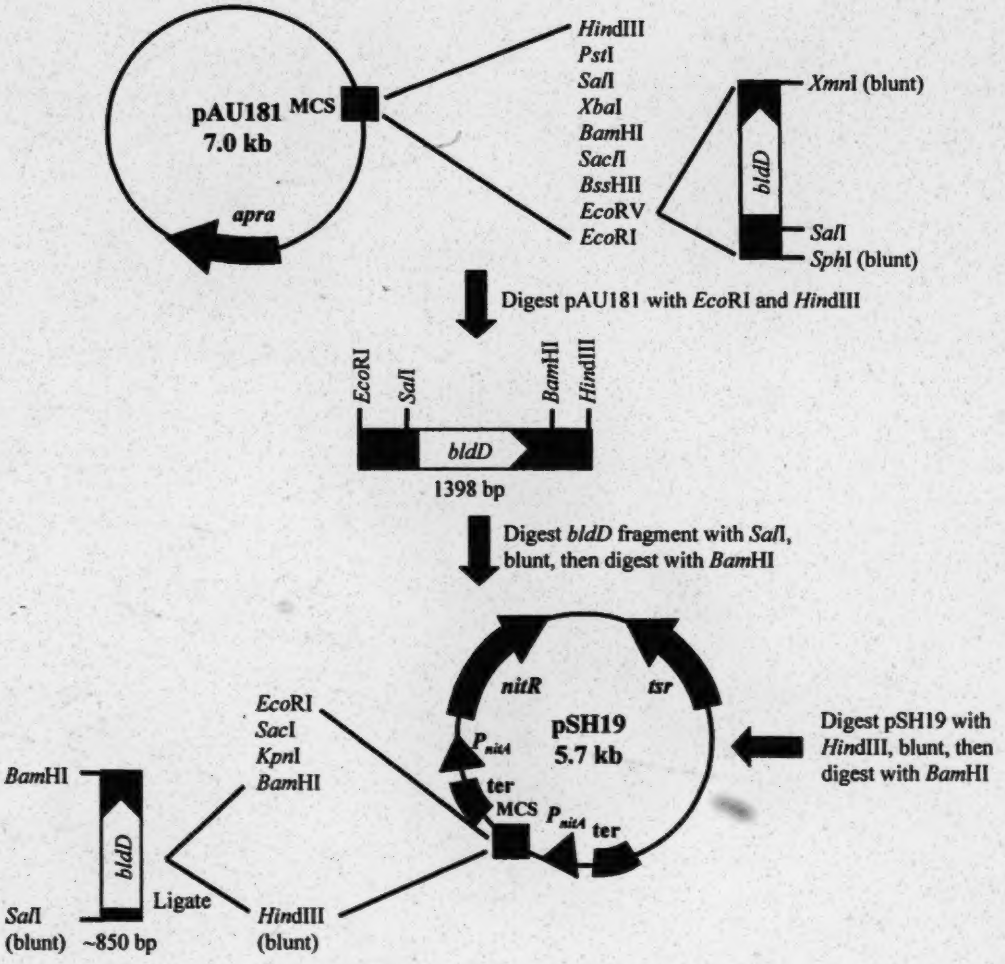

Flow-through
Wash
250 mM Nacl
500 mM NaCl
750 mM NaCl
1 M NaCl
1 M NaCl (off magnet)
Purified His6-BldD
Protein MWM
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
His6- BldD

170 bp *sigQ* promoter fragment are shown). This result was surprising because previous DNA-binding assays performed by C. Galibois (unpublished) showed that the His$_6$-BldD protein was capable of binding to the *sigQ* promoter region. Additionally, the *sigQ* promoter contains many putative BldD binding sites so it was expected that BldD would be able to bind very strongly to the *sigQ* promoter fragment.

It is possible that some cofactor protein or small effector molecule present only in *Streptomyces* extracts is necessary to mediate BldD binding to the *sigQ* promoter. To address this hypothesis, the same promoter fragments were used as bait to capture BldD protein from cell-free extracts harvested from *S. coelicolor* cultures inducibly expressing BldD or His$_6$-BldD protein (see Methods section 2.4.1.3 for details on protein induction and Results section 3.2.1 for details on construction of *S. coelicolor* His$_6$-BldD overexpression strains). However, neither the *sigQ* promoter fragment nor the *bldD* promoter fragment was able to capture the BldD or His$_6$-BldD protein from the *S. coelicolor* extracts (data not shown). In the case of the *bldD* promoter fragment, this result was not expected since the control experiment to purify His$_6$-BldD from *E. coli* JM109/pQE(BldD)$^+$ had been successful. Attempts to optimize the methodology involved increasing the binding reaction incubation time (from 90 minutes to 3 hours) to allow sufficient time for BldD-target DNA interactions. In addition, the total protein content was increased (from 4 mg to 15 mg) to increase the total amount of protein, and hence the amount of BldD, added to the binding reaction. Another reason for increasing the total protein content was because BldD or His$_6$-BldD expression levels from the P_{tipA} promoter in pIJ6902 in *S. coelicolor* have been observed to be much lower than that under P_{lac} in pQE9 in *E. coli* (Leskiw lab, unpublished). Despite repeated attempts to optimize the system, the BldD protein from *S. coelicolor* still could not be isolated using this methodology (data not shown).

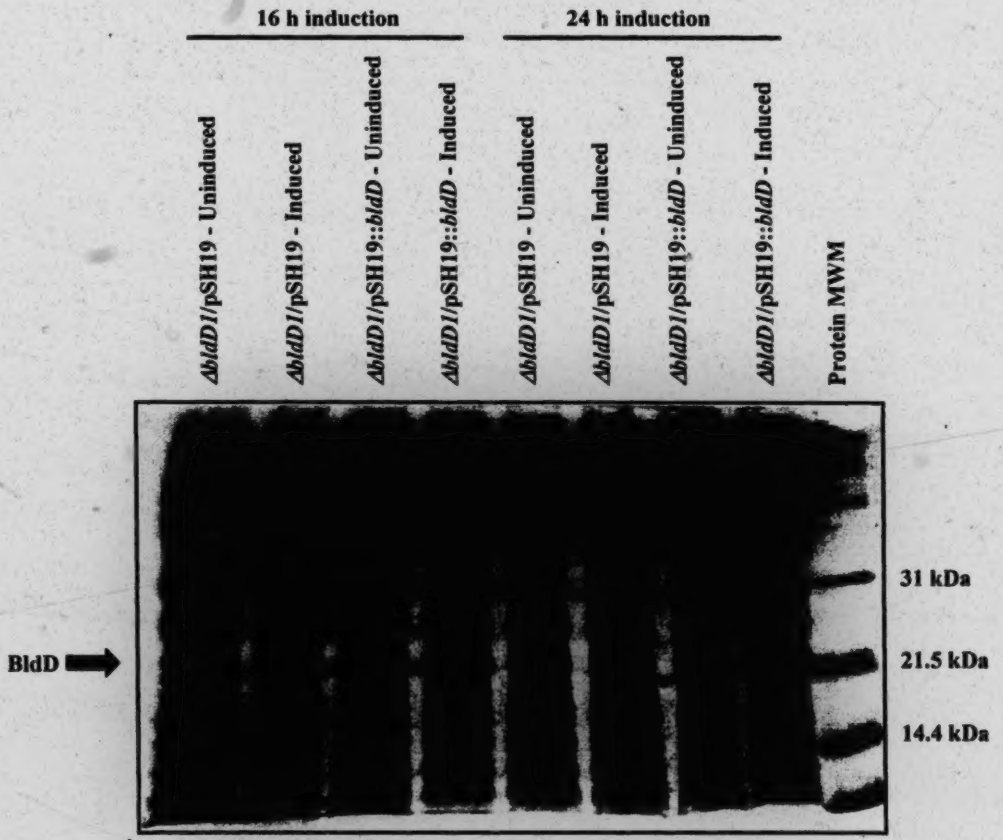
To further address the issue of low BldD expression under the P_{tipA} promoter, an alternate BldD expression vector was constructed. Construction of this expression vector used the hyper-inducible expression plasmid, pSH19 (Herai et al. 2004). This expression system is based on the expression of nitrilase (encoded by *nitA*), which requires the activity of the NitR positive transcriptional regulator, in *Rhodococcus rhodochrous* J1. In this system, NitR becomes activated following interaction with the ε-caprolactam inducer. The activated NitR protein can then bind the *nitA* promoter thereby resulting in expression of any protein of interest that is placed under the control of this promoter.

Construction of the pSH19::*bldD* expression plasmid involved liberating from pAU181 a 1398 bp *Eco*RI-*Hin*dIII fragment containing the *bldD* promoter region and coding sequence along with downstream flanking sequence (Table 2.2; Figure 3.15). Since there is a *Sal*I site located just upstream of the *bldD* start codon, the 1398 bp fragment was further digested with this restriction enzyme to release the *bldD* promoter region leaving behind a ~850 bp fragment containing the *bldD* coding region and downstream flanking sequence. This ~850 bp fragment was then treated with Klenow enzyme to remove cohesive overhangs and subsequently digested with *Bam*HI to facilitate ligation into the pSH19 polylinker. The pSH19 vector was digested with *Hin*dIII, treated with Klenow enzyme, and finally digested with *Bam*HI. Following ligation of the vector and *bldD* insert fragment, the ligation reaction was introduced into *ΔbldD1* protoplasts by transformation. *Streptomyces* colony hybridization was employed to screen 72 thiostrepton-resistant transformants for the presence of the *bldD* insert. One

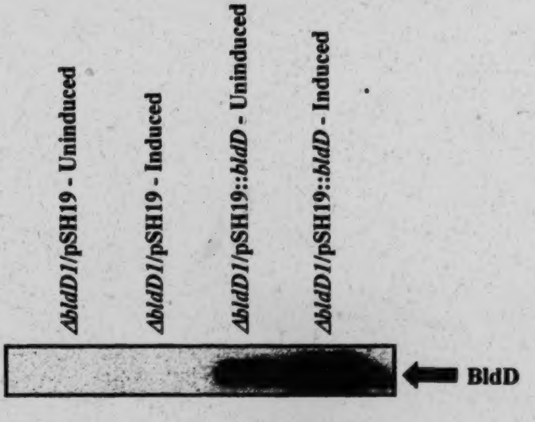

pAU181
7.0 kb
MCS
apra
HindIII
PstI
SalI
XbaI
BamHI
SacII
BssHII
EcoRV
EcoRI
XmnI (blunt)
bldD
SalI
SphI (blunt)
Digest pAU181 with EcoRI and HindIII
EcoRI
SalI
BamHI
HindIII
bldD
1398 bp
Digest bldD fragment with SalI, blunt, then digest with BamHI
nitR
tsr
pSH19
5.7 kb
P_nitA
ter
MCS
P_nitA
ter
Digest pSH19 with HindIII, blunt, then digest with BamHI
EcoRI
SacI
KpnI
BamHI
HindIII
(blunt)
Ligate
BamHI
bldD
SalI
(blunt)
~850 bp

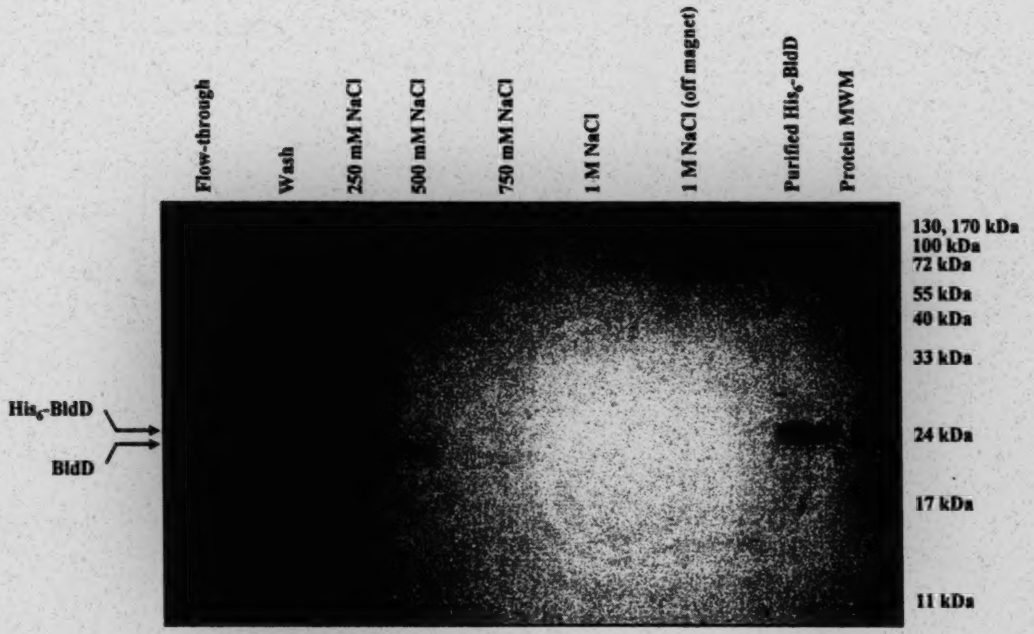
clone was confirmed to be positive for the *bldD* insert within pSH19 and was therefore subjected to sequence analysis to verify proper insertion of the fragment in the vector. Confirmation of BldD expression in the *ΔbldD1*/pSH19::*bldD* strain following addition of the ε-caprolactam inducer revealed very high overexpression of BldD protein, although the *nitA* promoter appeared very leaky as indicated by the high level of BldD expression in the uninduced control (Figure 3.16). The leaky expression could explain the lack of full complementation of the antibiotic and aerial mycelium negative, *bldD* phenotype when this strain was grown on solid R2YE medium supplemented with ε-caprolactam (data not shown). Since BldD is a key regulator of important developmental processes, the timing of BldD expression would be critical and therefore, uncontrolled, leaky overexpression of BldD would be detrimental for the strain. Despite the failed complementation, a decision was made to attempt to use the *bldD* and *sigQ* promoter fragments as bait to isolate the unmodified and modified BldD protein, respectively, from *ΔbldD1*/pSH19::*bldD* induced extract. It was hoped that this would address whether the DNA affinity methodology would work using *S. coelicolor* extracts expressing very high amounts of BldD protein. Binding reactions consisting of cell-free extract containing 4-5 mg total cellular protein and 6-10 μg biotinylated target DNA were incubated for three hours prior to addition of the streptavidin beads and another 30 minutes of incubation. After a single attempt, BldD was successfully purified using the *bldD* promoter fragment as bait (Figure 3.17), confirming the hypothesis that initial attempts to purify BldD from *S. coelicolor* using target DNA as bait were unsuccessful because BldD expression from the *tipA* promoter was much too low to isolate BldD in detectable amounts. There was no time to repeat this experiment and Western analysis was not performed to confirm that

(A) Detection of BldD expression by Coomassie staining


16 h induction
24 h induction
ΔbldD1/pSH19 - Uninduced
ΔbldD1/pSH19 - Induced
ΔbldD1/pSH19::bldD - Uninduced
ΔbldD1/pSH19::bldD - Induced
ΔbldD1/pSH19 - Uninduced
ΔbldD1/pSH19 - Induced
ΔbldD1/pSH19::bldD - Uninduced
ΔbldD1/pSH19::bldD - Induced
Protein MWM
BldD
31 kDa
21.5 kDa
14.4 kDa

(B) Detection of BldD expression by Western analysis


ΔbldD1/pSH19 - Uninduced
ΔbldD1/pSH19 - Induced
ΔbldD1/pSH19::bldD - Uninduced
ΔbldD1/pSH19::bldD - Induced
BldD


Flow-through
Wash
250 mM NaCl
500 mM NaCl
750 mM NaCl
1 M NaCl
1 M NaCl (off magnet)
Purified His6-BldD
Protein MWM
130, 170 kDa
100 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa
His6-BldD
BldD

the purified protein was BldD. Although the results for the *bldD* promoter fragment were promising, attempts to isolate the modified BldD protein using this methodology were eventually abandoned because the use of the *sigQ* promoter fragment (only the 170 bp LBU1-CGA41 fragment was tried) did not result in affinity purification of any form of BldD (data not shown). Whether BldD could be detected by Western analysis following affinity purification was not addressed. It is possible that the method had been somewhat successful and had only required higher protein content in the binding reaction to facilitate purification of BldD in detectable amounts. The inability to capture BldD protein with the *sigQ* promoter fragment as bait could be because the binding of BldD to the *sigQ* promoter region requires the presence of some cofactor protein and the ratio of BldD to this cofactor protein might somehow be critical in dictating whether binding can or cannot occur. Furthermore, it is possible that BldD modification cannot occur when BldD is overexpressed to such a high degree. Since it is expected that BldD binds to the *sigQ* promoter in its modified form, the lack of BldD modification would render the *sigQ* promoter fragment useless for BldD capture.

3.2. Studies to isolate and characterize BldD-binding partner(s)

BldD belongs to the same family of transcriptional regulators as SinR, a *B. subtilis* regulatory protein involved in many important processes including sporulation (Gaur et al. 1991). The SinR repressor activity is abolished when bound by SinI, a SinR antagonist (Bai et al. 1993). Therefore, it is possible that BldD function might also be regulated through protein-protein interactions. The repressor function of BldD might be abolished when BldD interacts with a protein partner, which changes the BldD conformation such that it can no longer bind to promoter/operator regions of target genes.

Alternatively, the switch from the BldD repressor form to the activator form could involve interaction with a protein partner. As stated above in section 3.1.5, this could be one explanation for the failure to isolate BldD bound to the *sigQ* promoter region.

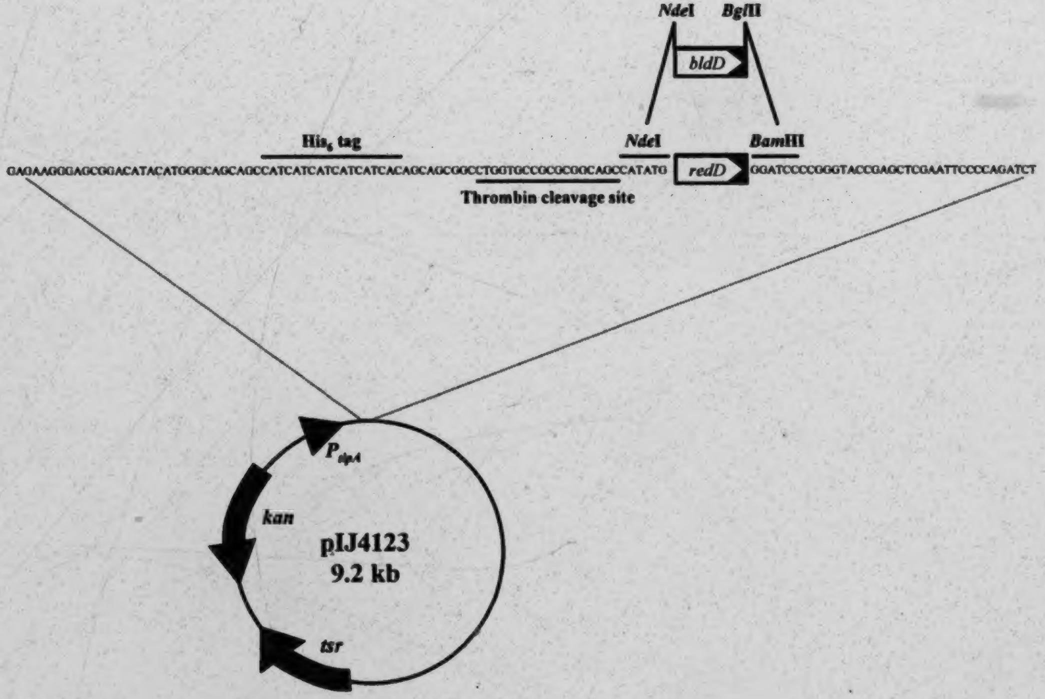
3.2.1. Construction of His$_6$-BldD overexpression system

In order to try and identify a BldD-binding partner(s), it was necessary to explore a technique that would allow for co-purification of BldD and any protein(s) bound to it. The creation of an expression system for overexpression of BldD in *S. coelicolor* as a histidine-tagged protein would allow for purification of BldD, and any bound protein partner(s), from cell-free extracts. Nickel affinity chromatography, a well-characterized purification system, would allow for efficient, one step, isolation of His-tagged BldD and any bound protein partner(s). Furthermore, since many protein-protein interactions are either weak or transient, the use of chemical crosslinkers, prior to the affinity chromatography, would increase the likelihood of capturing BldD-binding partner(s).

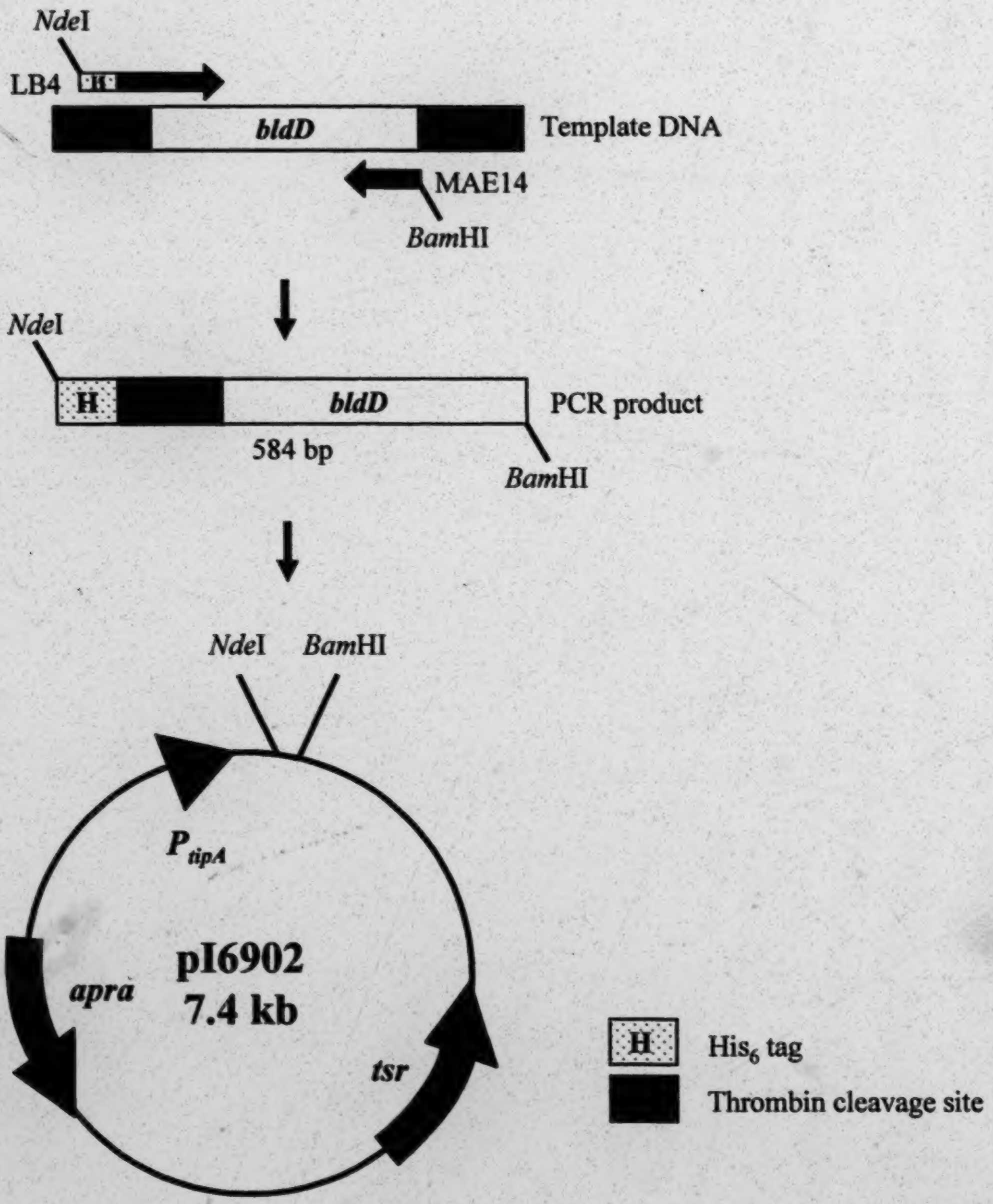
Initial attempts to create an expression system for *in vivo* overexpression of His$_6$-BldD protein involved the use of a high-copy *Streptomyces* plasmid containing a thiostrepton-inducible *tipA* promoter (Takano et al. 1995). This vector, called pIJ4123, has the advantage of allowing for good selection of positive transformants. pIJ4123 contains a cloned *redD* (regulator for red pigmented antibiotic production) fragment in the polylinker so that transformants containing this plasmid overproduce red undecylprodigiosin pigment giving the colonies an intense red colour. Replacement of the *redD* fragment with a gene of interest would result in the production of white/cream transformants.

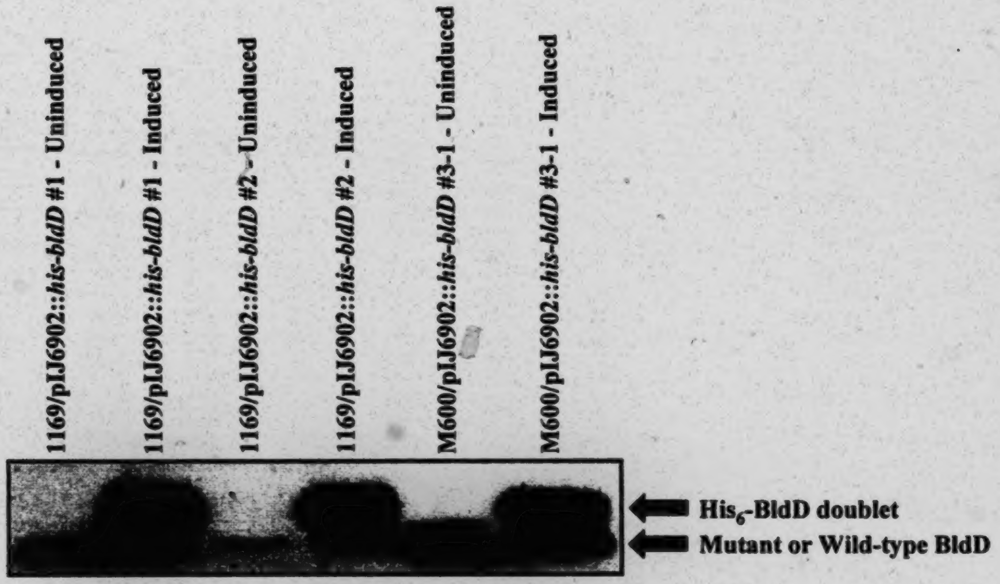
The cloning strategy involved replacing the *redD* gene on an *Nde*I-*Bam*HI fragment with an ~850 bp *Nde*I-*Bgl*II fragment (liberated from pAU243; see Table 2.2), which contains the *bldD* coding region and ~300 bp downstream sequence (Figure 3.18). Following ligation of the *Nde*I-*Bgl*II fragment into the pIJ4123 polylinker, the ligation mix was introduced both into the 1169 *bldD* point mutant strain and into the *ΔbldD1* mutant strain by protoplast transformation. Although several cloning attempts were undertaken, only a total of six cream/pale orange transformants were obtained. Most attempts only produced red colonies or no colonies at all. The cream/pale orange transformants were promising because they appeared to partially complement (some aerial hyphae production could be seen) the *bldD* mutant phenotype when grown in the presence of thiostrepton, which should have induced the expression of the BldD fusion protein. Surprisingly, however, confirmation of the suspected positive clones by PCR using *bldD*-specific primers did not detect the *bldD* insert, and Western analysis failed to detect BldD overexpression following thiostrepton induction (data not shown). It is possible that the cream colonies gained this phenotype due to the spontaneous loss of the *redD* fragment rather than the replacement of the *redD* fragment with the *bldD* fragment.

A second approach to constructing an expression plasmid for His$_6$-BldD overexpression involved the use of pIJ6902, a low-copy expression plasmid capable of site-specific integration into the *Streptomyces* chromosome (Huang et al. 2005). One major advantage of working with pIJ6902 is that this plasmid can be propagated in *E. coli* making it much easier and faster to clone and screen for positive transformants. The cloning strategy involved introducing into pIJ6902 a PCR product that contains the His$_6$ sequence followed by a thrombin cleavage sequence (in case we want to release BldD


*Nde*I
*Bgl*II
bldD
His$_6$ tag
*Nde*I
*Bam*HI
GAGAAGGGAGCGGACATACATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATG
redD
GGATCCCCGGGTACCGAGCTCGAATTCCCCAGATCT
Thrombin cleavage site
P_{tipA}
kan
pIJ4123
9.2 kb
tsr

from the His$_6$ tag) upstream of the *bldD* gene (Figure 3.19). To create this PCR product, a long oligonucleotide primer was synthesized to include a 5' *Nde*I restriction site and all of the His$_6$/thrombin sequence (from pIJ4123) followed by the first 22 nucleotides of the *bldD* gene. Since the start codon of *bldD* overlaps with a naturally-occurring *Nde*I site (CATATG), the first "T" in the recognition site was mutagenized to a "C", making it possible to later digest the resulting PCR fragment with *Nde*I without releasing the His$_6$/thrombin sequence from the sequence corresponding to the *bldD* gene. This primer, named LB4, was used with the primer MAE14, which contains a 5' *Bam*HI tail, to produce a 584 bp PCR product. After successful insertion of the *Nde*I-*Bam*HI-digested PCR product into the polylinker of the similarly-digested pIJ6902 vector, the plasmid was passaged through the non-methylating *E. coli* strain ET12567, and then transferred into *S. coelicolor* wild-type M600 and 1169 *bldD* point mutant (the markerless *ΔbldD* null strain had not been available at the time of these experiments) strains by conjugation. Once apramycin-resistant colonies had been isolated, chromosomal DNA was isolated and subjected to PCR and sequence analysis to confirm proper integration of the pIJ6902::*his-bldD* construct into the chromosome of each *S. coelicolor* transconjugant (data not shown). Although Western analysis showed that His$_6$-BldD protein was being expressed, an additional slightly smaller band was also detected (Figure 3.20). This smaller band may simply represent a degradation fragment of the His$_6$-BldD protein. Another possibility is that slippage had occurred during transcription and that some tagged BldD forms might lack one or two His residues and therefore might migrate faster due to the smaller molecular weight. Subsequent attempts to purify the His$_6$-BldD protein by nickel affinity chromatography were unsuccessful as no distinct bands


NdeI
LB4
H
bldD
Template DNA
MAE14
BamHI
NdeI
H
bldD
PCR product
584 bp
BamHI
NdeI
BamHI
P_tipA
pI6902
7.4 kb
apra
tsr
H
His6 tag
Thrombin cleavage site


1169/pIJ6902::*his-bldD* #1 - Uninduced
1169/pIJ6902::*his-bldD* #1 - Induced
1169/pIJ6902::*his-bldD* #2 - Uninduced
1169/pIJ6902::*his-bldD* #2 - Induced
M600/pIJ6902::*his-bldD* #3-1 - Uninduced
M600/pIJ6902::*his-bldD* #3-1 - Induced
His$_6$-BldD doublet
Mutant or Wild-type BldD

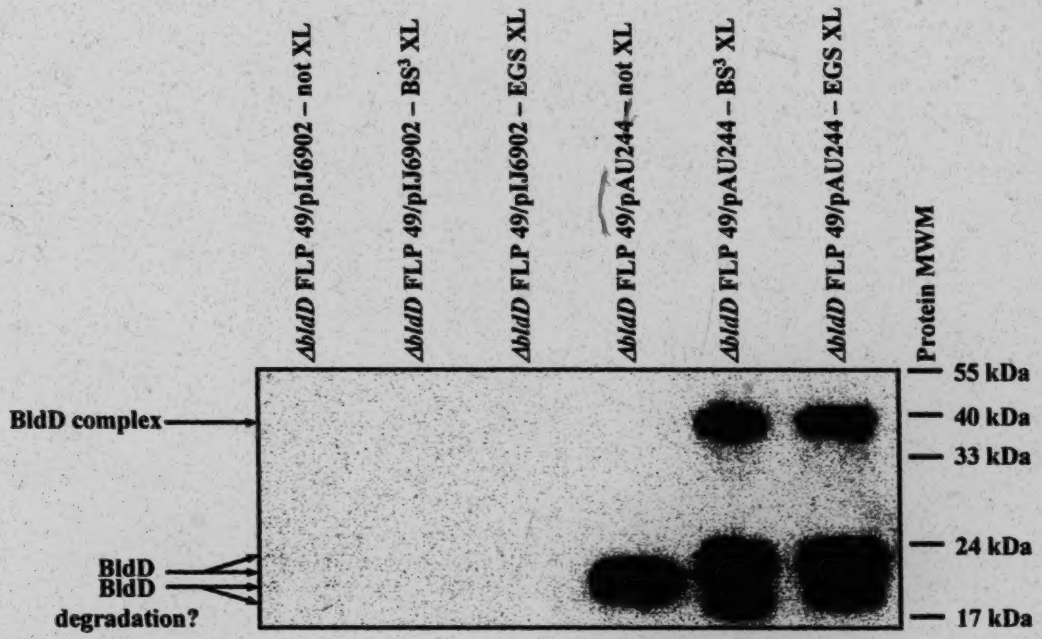
corresponding to the molecular weight sizes of either of the His_6-BldD isoforms could be visualized in any of the wash fractions following electrophoresis and Coomassie staining of the gel (data not shown). Therefore, experiments to overexpress BldD as a histidine-tagged protein were no longer pursued. One explanation for the lack of success in purifying His_6-BldD protein could be due to the very low level expression of the fusion protein from the *tipA* promoter. Furthermore, if slippage had occurred and caused the production of BldD fusion protein containing fewer His residues, the binding affinity of His-tagged BldD to the nickel column would be reduced thereby resulting in lower yields of purified His-tagged BldD. Experiments carried out to purify His_6-BldD protein (two attempts) only used cell-free extracts from 100 mL cultures and it was not explored whether larger volumes of culture would have allowed for successful purification of His_6-BldD protein. Successful protein purification often requires scale-ups of the culture volumes because an intense protein band detected by Western analysis does not necessarily mean the protein is present at abundant levels.

3.2.2. Chemical crosslinking studies

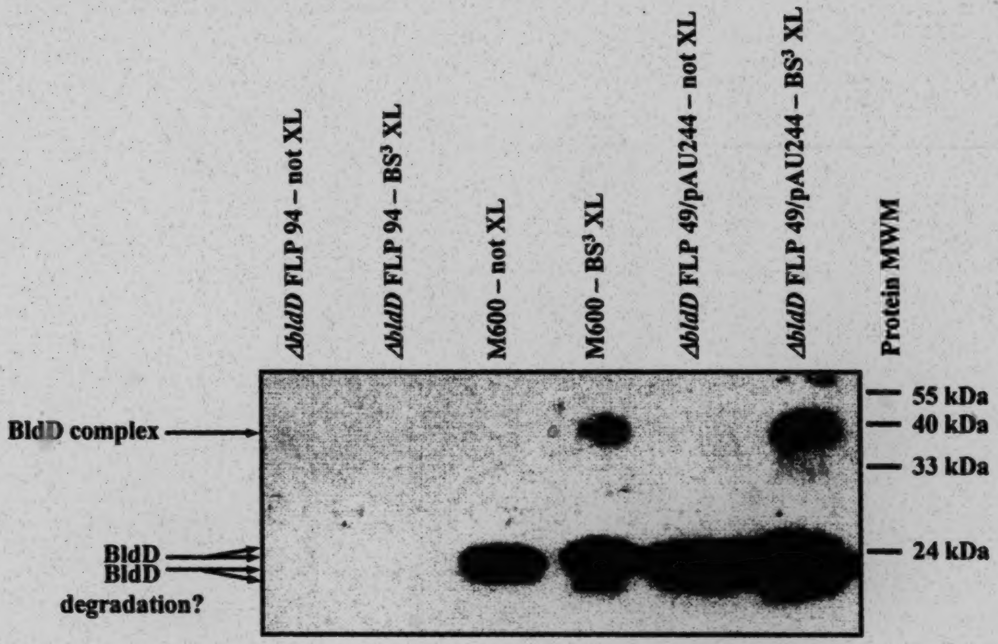
Although a His_6-BldD overexpression system did not yield sufficient levels of expression for small scale one-step purification of His_6-BldD, a decision was made to go ahead with the chemical crosslinking experiments, with the intention that immunoprecipitation would be used, in place of nickel affinity chromatography, to isolate BldD and any BldD-binding partner(s). *In vitro* chemical crosslinking experiments were performed following preparation of total cell lysate using a gentle lysis method (to prevent disruption of protein interactions) involving enzymatic digestion of the cell wall to form protoplasts (see Methods section 2.2.5.5). Once formed, the protoplasts were osmotically-lysed prior to addition of chemical crosslinkers. Crosslinking experiments were done using the *S. coelicolor* wild-type strain M600 and, as a negative control, the *ΔbldD* FLP 94 strain. To increase the chances of detecting BldD complexes, chemical crosslinking experiments were also done using the *ΔbldD* FLP 49/pAU244 strain inducibly overexpressing BldD, together with the control *ΔbldD* FLP 49 strain harbouring the parent pIJ6902 plasmid. As an additional control, crosslinked samples were compared to uncrosslinked samples. Two different chemical crosslinkers (only one crosslinker was tried with M600 and *ΔbldD* FLP 94) were used, both at a final reaction concentration of 1 mM. BS^3 and EGS, having a spacer length of 11.4 Å and 16.1 Å, respectively, are both amine-reactive, homobifunctional crosslinkers (Sinz 2003). One advantage of using EGS is that it possesses a cleavable spacer arm giving the possibility of crosslink reversal.

Western analysis of cell-free extracts detected a higher molecular weight product in both the wild-type and BldD overexpression strain (although much fainter in the former) when treated with the crosslinkers while there was no complex detected in the uncrosslinked samples (Figure 3.21A, B). Similar results were seen for both crosslinkers and the experiment was performed only once (only BS^3 crosslinker was tried with M600 and *ΔbldD* FLP 94). The lack of complex formation in the negative control strains, *ΔbldD* FLP 94 and the *ΔbldD* FLP 49 with the parent pIJ6902 vector, suggests that the complex was BldD-dependent. It appears that the addition of the crosslinkers had affected the migration of the BldD protein and the probable BldD degradation fragment since the molecular weight sizes of these bands in the crosslinked sample differed from those in the uncrosslinked sample. While the increase in size of the BldD band could be

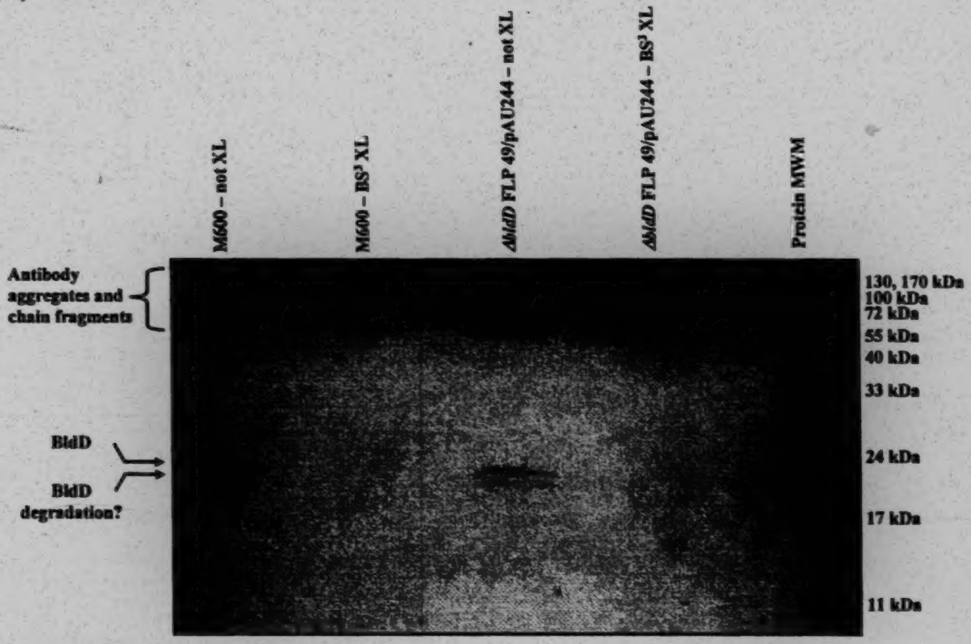
(A) Complex formation in BldD overexpression strain

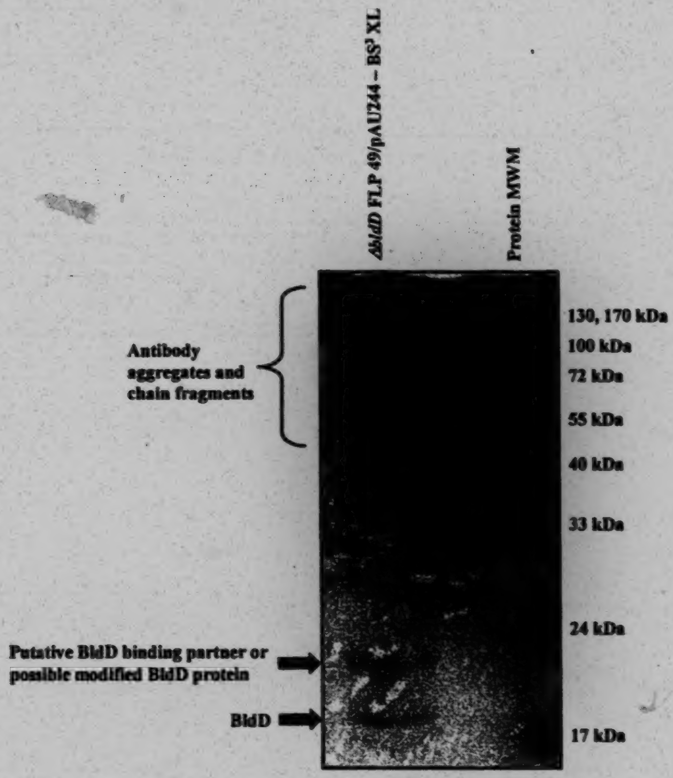

ΔbldD FLP 49/pIJ6902 – not XL
ΔbldD FLP 49/pIJ6902 – BS^3 XL
ΔbldD FLP 49/pIJ6902 – EGS XL
ΔbldD FLP 49/pAU244 – not XL
ΔbldD FLP 49/pAU244 – BS^3 XL
ΔbldD FLP 49/pAU244 – EGS XL
Protein MWM
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
BldD complex
BldD
BldD
degradation?

(B) Complex formation in M600 wild-type strain


ΔbldD FLP 94 – not XL
ΔbldD FLP 94 – BS^3 XL
M600 – not XL
M600 – BS^3 XL
ΔbldD FLP 49/pAU244 – not XL
ΔbldD FLP 49/pAU244 – BS^3 XL
Protein MWM
55 kDa
40 kDa
33 kDa
24 kDa
BldD complex
BldD
BldD
degradation?

due to the nonspecific crosslinking of BldD to some small peptide fragment, the reason for the decrease in the size of the probable BldD degradation fragment is unclear. Nevertheless, these results were deemed not significant and attention was instead focused on characterizing the BldD-dependent complex. Although the size of the higher molecular weight product appeared to correspond to the size of the BldD dimer (~36 kDa), it was possible that it might also be BldD monomer bound to a binding partner and therefore IP experiments were used to try to capture the complex for mass spectrometric analysis to determine the identity of protein components. Uncrosslinked and BS^3-crosslinked total cell lysates purified from the M600 and *ΔbldD* FLP 49/pAU244 (induced) strains were subjected to immunoprecipitation, followed by SDS-PAGE on a 15% SDS-PAG and Coomassie staining. Unexpectedly, no complex could be observed in the immunoprecipitated crosslinked samples (Figure 3.22A). The band corresponding to BldD monomer, which was present in the uncrosslinked samples, was absent in the crosslinked samples, suggesting that BldD monomer was being sequestered in a protein complex, although no band corresponding to that BldD-containing complex could be visualized. It was unclear how the BldD monomer band could be relatively intense in the uncrosslinked samples and yet be undetectable after the addition of crosslinker, especially since Western analysis had detected BldD monomer in the crosslinked samples (see again, Figure 3.21A, B). Perhaps some of the BldD monomer is sequestered in complexes that have formed larger aggregates that are unable to enter the gel. The remaining BldD monomers may not be detectable by Coomassie staining because their levels are now below the protein detection limit. Another possibility is that intramolecular or intermolecular crosslinking resulting from BldD interacting with itself


(A) Small scale IP
M600 – not XL
M600 – BS^3 XL
ΔbldD FLP 49/pAU244 – not XL
ΔbldD FLP 49/pAU244 – BS^3 XL
Protein MWM
Antibody aggregates and chain fragments
BldD
BldD degradation?
130, 170 kDa
100 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa
11 kDa


(B) Large scale IP
ΔbldD FLP 49/pAU244 – BS^3 XL
Protein MWM
Antibody aggregates and chain fragments
Putative BldD binding partner or possible modified BldD protein
BldD
130, 170 kDa
100 kDa
72 kDa
55 kDa
40 kDa
33 kDa
24 kDa
17 kDa

or another protein, respectively, had reduced the number of BldD epitopes available for binding by αBldD antibodies. However, the fact that both the BldD monomer and the BldD-dependent complex could be visualized by Western detection suggests that only a small number of epitopes are needed to visualize protein by Western analysis while more epitopes are required to precipitate large enough quantities of BldD to visualize by Coomassie staining.

After three unsuccessful attempts at purifying the BldD-dependent complex, a larger scale IP experiment were performed to increase the chances of detecting the BldD complex by Coomassie staining. For this experiment, which was performed only once, immunoprecipitated proteins from 12 reactions, with each reaction containing 2.5 mg total protein content from the BS^3-crosslinked *ΔbldD* FLP 49/pAU244 strain (induced), were pooled and analyzed on a large SDS-PAG. This attempt also failed to isolate the previously observed BldD complex, although a band migrating at ~22 kDa was observed (Figure 3.22B). This band was excised and identified by LC MS/MS. Disappointingly, it was found that the band represented BldD protein still attached to antibody contaminating fragments. As stated above, it is possible that BldD complex formation, or even intramolecular crosslinking of the BldD monomers, somehow resulted in steric interferences that prevented efficient binding of the αBldD antibodies to BldD protein. The presence of the chemical crosslinker could have also contributed to the steric interferences. The possibility that crosslink reversal of the EGS-crosslinked samples and subsequent immunoprecipitation of the de-crosslinked samples might have allowed for detection of the BldD complex was not explored. Although Western analysis was sensitive enough to detect the BldD complex, it is possible that there was so little immunoprecipitated BldD complex that Coomassie staining could not detect the complex. Whether more sensitive stains, such as silver staining or Sypro® Ruby (Bio-Rad) staining could detect the BldD complex was also not explored.

3.3. Studies to confirm that *sigQ* is an activated target of BldD

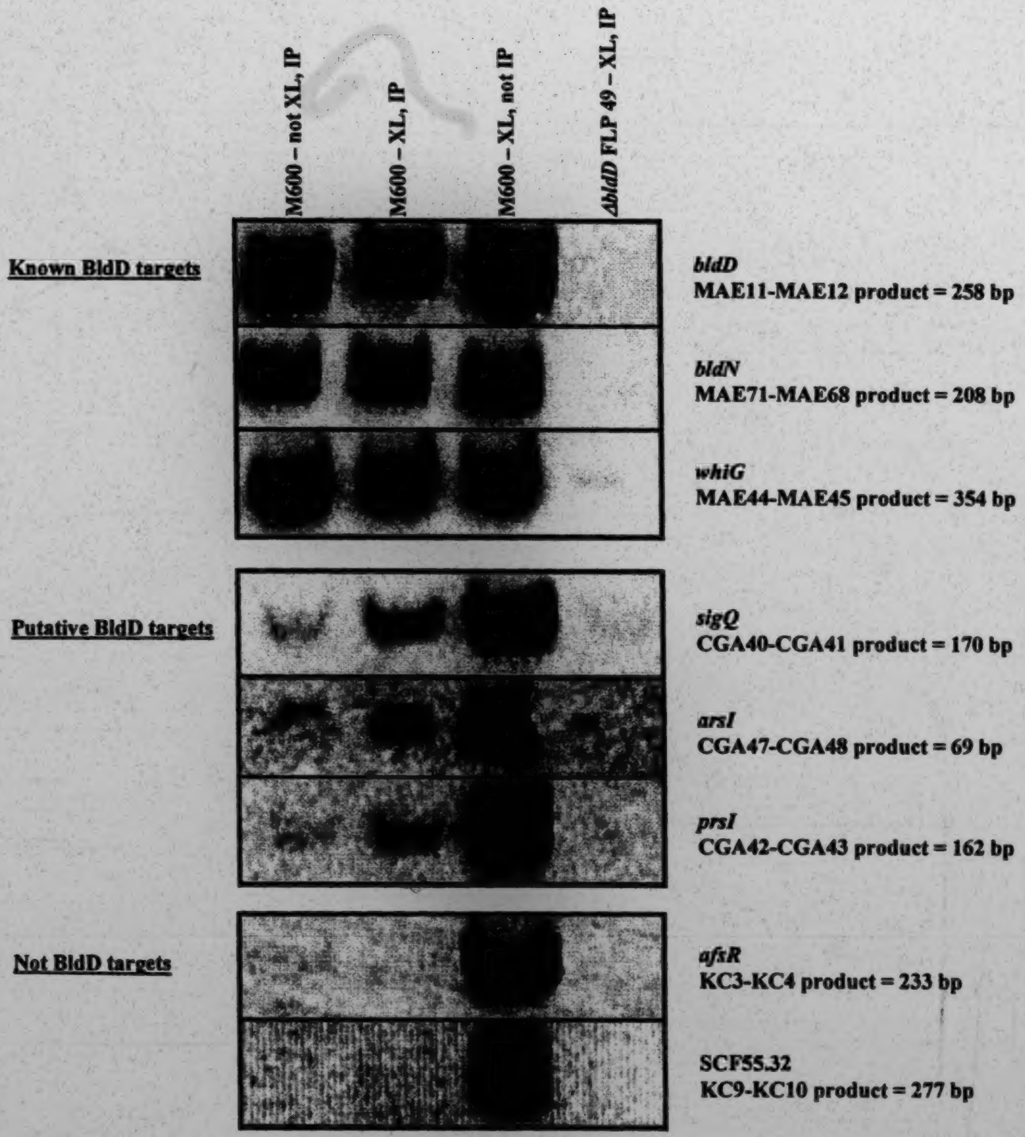
Since attempts to isolate either a modified BldD protein or a BldD-binding partner had been unsuccessful, the decision was made to focus attention on studies to confirm that *sigQ* is a BldD target. The purpose of these studies was to provide stronger support for the role of BldD as an activator of gene expression. As stated earlier in section 3.1, previous microarray work identified the *sigQ* gene to be a potential BldD target whose expression is activated when *bldD* is induced (C. Galibois, unpublished). Although DNA-binding studies confirmed that BldD could bind to the *sigQ* promoter region *in vitro* (C. Galibois, unpublished), no additional experiments were done to authenticate the microarray results. Therefore, as part of this study, experiments were performed to confirm and complement the results of the microarray and DNA-binding studies.

3.3.1. Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed as an *in vivo* method to confirm the initial DNA-binding studies that revealed that BldD could bind to the *sigQ* promoter region *in vitro* (C. Galibois, unpublished). The ChIP technique has been successful for the identification and characterization of protein-DNA interactions (Solomon and Varshavsky 1985; Jakimowicz et al. 2002; Shin and Groisman 2005). This technique involves the stabilization of protein-DNA complexes *in vivo* by using the highly efficient, membrane-permeable chemical crosslinker, formaldehyde. The cells are then lysed by sonication and the DNA is sheared to smaller fragments (500-2000 bp). The protein with the attached DNA is immunoprecipitated followed by heat treatment to reverse the protein-DNA crosslinks. The immunoprecipitated DNA is then subjected to PCR analysis to amplify suspected target sequences of the protein.

In this study, *S. coelicolor* strains (see below) were grown to high cell density (late exponential) and formaldehyde was then used to crosslink BldD to target DNA sequences. The cells were lysed by sonication and DNA was sheared to smaller fragments. Initially sonication had been used to shear the chromosomal DNA because the majority of the published procedures employ this technique. However, in this study the use of sonication did not mediate sufficient shearing of the DNA, which resulted in precipitation of BldD target sequences (ie. chromosomal DNA) in ChIP samples where no BldD was present (*ΔbldD* FLP 49 mutant) to bind to the target sequences (data not shown). Although no BldD is present in the *ΔbldD* null strain, meaning that there should be no precipitation of BldD target sequences, contamination of ChIP samples with nonspecific DNA is common for the ChIP procedure, due to the large number of centrifugation steps, and therefore the presence of larger DNA fragments caused by insufficient shearing resulted in the increase in probability of amplifying BldD target sequences. Although repeated attempts were made to increase the degree of DNA shearing by increasing the sonication time and intensity (3 X 20 second pulses on output 1 to 12 X 15 second pulses on output 4), the ChIP results remained unchanged. Finally, the use of an enzyme cocktail purchased from Active Motif resulted in successful shearing of DNA to fragments of 500 – 2000 bp (data not shown). DNA fragments bound to BldD protein were then immunoprecipitated and the immunocomplexes were then captured with protein A Sepharose. The following ChIP DNA samples were prepared: (1) M600 wild-type strain – not crosslinked and immunoprecipitated [uncrosslinked negative control]; (2) M600 wild-type strain – crosslinked and immunoprecipitated [test sample]; (3) M600 wild-type strain – crosslinked but not immunoprecipitated [total DNA positive control]; (4) *ΔbldD* FLP 49 strain – crosslinked and immunoprecipitated [no BldD negative control]. An equal amount of each ChIP DNA sample was used in semi-quantitative (24 cycles) PCR reactions with primer pairs amplifying target regions containing known or putative BldD binding sites (~70-350 bp). As an additional negative control, PCR reactions were performed with primer pairs amplifying promoter regions of genes that are not expected to be BldD targets. For all PCR reactions, it was expected that there would be a PCR product in the total DNA positive control because it contains the population of all input DNAs. Three independent ChIP experiments were performed and representative PCR results are shown (Figure 3.23).

PCR reactions were first performed using primer pairs to confirm enrichment of known BldD target DNAs in the test ChIP sample. BldD is known to bind to the promoter regions of *bldD*, *bldN*, and *whiG* to repress the transcription of these genes (Elliot and Leskiw 1999; Elliot et al. 2001). Enrichment of the *bldD*, *bldN*, and *whiG* promoter regions was detected with the primer pairs MAE11-MAE12, MAE71-MAE68, and MAE44-MAE45, respectively. The results for all three genes were the same. PCR products were detected in all ChIP samples except for the no BldD negative control (Figure 3.23). It was expected that there would be no PCR product in the no BldD negative control since there is no BldD protein to be immunoprecipitated and therefore no DNA should be isolated. It was also expected that there would be an intense PCR


M600 – not XL, IP
M600 – XL, IP
M600 – XL, not IP
ΔbldD FLP 49 – XL, IP
Known BldD targets
bldD
MAE11-MAE12 product = 258 bp
bldN
MAE71-MAE68 product = 208 bp
whiG
MAE44-MAE45 product = 354 bp
Putative BldD targets
sigQ
CGA40-CGA41 product = 170 bp
arsI
CGA47-CGA48 product = 69 bp
prsI
CGA42-CGA43 product = 162 bp
Not BldD targets
afsR
KC3-KC4 product = 233 bp
SCF55.32
KC9-KC10 product = 277 bp

product in the test samples since *bldD*, *bldN*, and *whiG* are all known to be BldD targets. The presence of a PCR product in the uncrosslinked negative control was, however, not expected since DNA was not crosslinked to BldD protein and was therefore not expected to remain stably bound to BldD after the sonication employed for cell lysis. It was anticipated that crosslinking would be required to stabilize the BldD-DNA complexes and allow immunoprecipitation of BldD target DNAs. Even more surprising is that the PCR product in the uncrosslinked negative control is as intense as the test sample, suggesting that BldD normally binds very strongly to these target promoter regions and crosslinking is not needed to stabilize the protein-DNA interaction. However, it should be noted that the majority of the ChIP procedures described in the literature employ sonication as a means for cell lysis and DNA shearing. In these procedures the sonication times are often much longer and these conditions likely ensure the disruption of any protein-DNA interactions that are not stabilized by chemical crosslinking.

Next, PCR reactions were performed with primer pairs amplifying the promoter region of *sigQ*. The primer pair CGA40-CGA41 was used to amplify the *sigQ* promoter region. Additionally, PCR reactions using primer pairs amplifying promoter regions of two other putative BldD target genes (C. Galibois, unpublished) were performed to give a comparison for the *sigQ* results. The primer pairs CGA47-CGA48 and CGA42-CGA43 were used to amplify the promoter regions of *arsI* and *prsI*, respectively. From the results, all three genes appear to be weak BldD targets since DNAs containing the promoter regions of these genes did not appear to be highly enriched in the test sample as indicated by the faint PCR band in these ChIP samples (Figure 3.23). For all three genes, there was also a very faint PCR product in the uncrosslinked negative control, suggesting that BldD binding to the target region was strong enough that crosslinking was not needed to stabilize the interaction. This result, however, contradicts the result that these target DNAs are not well enriched in the test sample, a result that suggests that BldD binding to these promoter regions is not very strong. In the case of *sigQ*, weak binding was not expected since the *sigQ* promoter region contains many putative BldD binding sites (17 half repeats; see again, Figure 3.13A, B) and *sigQ* expression was very strongly activated (~20 fold) when microarray analyses were used to address global gene expression after *bldD* induction (C. Galibois, unpublished). The multiple binding sites, together with the strong upregulation, suggest that BldD should be able to bind to the *sigQ* promoter region with high affinity.

Finally and as negative controls, PCR reactions were performed with primer pairs amplifying promoter regions of genes that are not expected to be BldD targets. For these PCRs, it was expected that the total DNA positive control would have a PCR product while all other ChIP samples would not. The primer pairs KC3-KC4 and KC9-KC10 were used to amplify the promoter regions of *afsR* and SCF55.32 (putative BldG target), respectively. As expected, for both genes, a PCR product was detected only in the total DNA positive control (Figure 3.23).

In conclusion, the ChIP analyses showed that BldD was capable of binding to the *sigQ* promoter region *in vivo*, although the binding affinity appeared weak. All ChIP experiments were done using cells grown to late exponential-phase (as judged by high cell density growth just prior to pigmented antibiotic production) and therefore it is possible that BldD binding to the *sigQ* promoter region occurs only at a very specific time in development and these cells had not been used in the ChIP analyses. The cultures used in the microarray analyses mentioned above were induced at an OD_{600} of ~0.6 which represents early-exponential phase. The differences in culture conditions could account for the discrepancy between the ChIP results presented here and the microarray results obtained by C. Galibois.

3.3.2. Analysis of *sigQ* expression

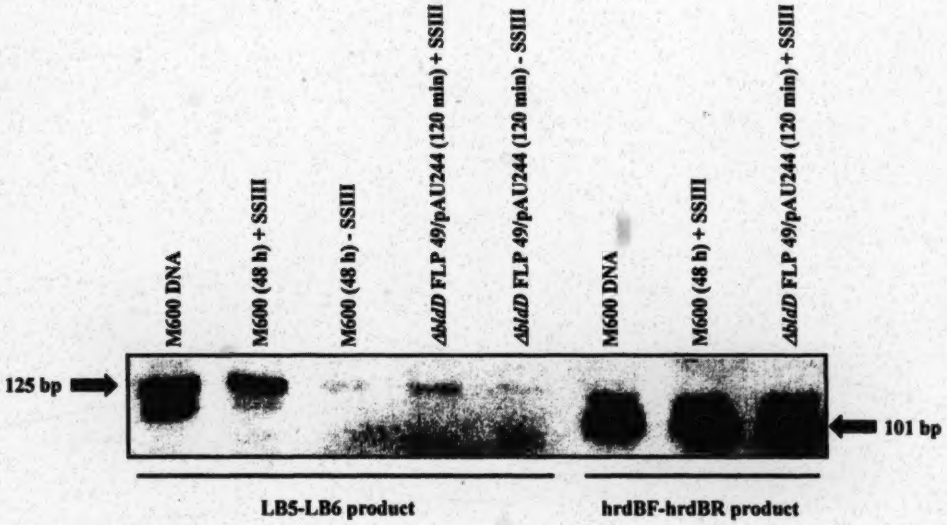
Since the ChIP experiments had suggested that BldD may not bind strongly to the *sigQ* promoter region, a decision was made to use the RT-PCR technique to confirm the initial microarray analyses that had shown BldD dependence of *sigQ* expression (C. Galibois, unpublished). Real-time RT-PCR was employed to compare *sigQ* expression in the *S. coelicolor* wild-type strain to the *ΔbldD* FLP 49 strain and in the BldD overexpression strain to the vector control strain. A solid medium RNA time course (with time points corresponding to stages of vegetative growth, the transition between vegetative and aerial mycelium growth, aerial mycelium formation, the transition between aerial mycelium formation and sporulation, and sporulation) was prepared for the *S. coelicolor* wild-type strain M600. The same time points were used for a solid medium RNA time course of the aerial mycelium and sporulation defective *ΔbldD* FLP 49 strain. A liquid medium induction time course (0 min, 15 min, 30 min, 45 min, 60 min, and 120 min) was prepared for the *ΔbldD* FLP 49 strain harbouring pAU244 (where *bldD* is under inducible control from the P_{tipA} promoter) and the *ΔbldD* FLP strain harbouring the parent pIJ6902 vector. RNA was isolated from each time point and first-strand cDNA was then synthesized using the SuperScript™ III first-strand synthesis kit (Invitrogen). The primer pair LB5-LB6 was designed to amplify from the *sigQ* cDNA a 125 bp PCR product homologous to the *sigQ* 5' coding sequence (+45 to +169 with respect to the *sigQ* transcription start point). As a control for RNA levels, detection of cDNA made from transcripts of *hrdB*, encoding the vegetative housekeeping RNA polymerase, was performed. The primer pair hrdBF-hrdBR was used to amplify a 101 bp PCR product homologous to the *hrdB* 5' coding sequence (+125 to +225 with respect to the *hrdB* translation start point).

The first attempt to synthesize cDNA used gene-specific primers to synthesize first-strand cDNA from the M600 (24 hour) sample. LB6 and hrdBR (the downstream reverse primers) were used for synthesis of *sigQ* and *hrdB* cDNA, respectively. Reverse transcriptase reactions (RT reactions) containing either no SuperScript™ III (SSIII) or no RNA template were performed to check for DNA contamination and reagent quality, respectively. Before doing the real-time RT-PCR experiment, RT reactions were used in end-point PCR reactions (25 cycles) to ensure successful synthesis of cDNA. A PCR reaction containing denatured M600 genomic DNA was included to check PCR conditions. Faint bands were seen in the PCR reactions containing RT reactions with no SSIII, indicating slight DNA contamination in the RNA sample (data not shown). The absence of product in the PCR reactions containing RT reactions with no RNA template indicates that all RT-PCR reagents are good quality. Finally, product was detected for *hrdB* but not for *sigQ*, however, since *sigQ* was barely amplifiable from the genomic DNA control, it was assumed that the *sigQ* PCR was just not as efficient. Therefore, the end-point PCR using the same RT reactions was repeated with the cycle number increased to 28 cycles. Again, intense PCR products were detected for *hrdB* (data not shown). Although more LB5-LB6 product could be amplified from the genomic control with the increased cycle number, PCR product was barely detectable in PCR reactions with RT reactions containing SSIII.

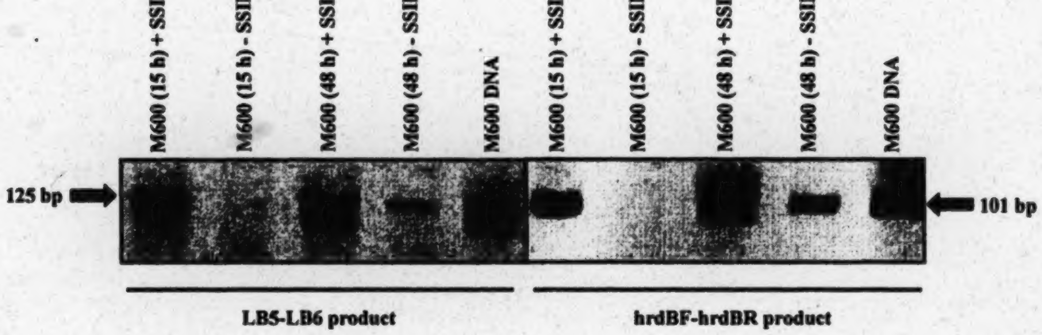
Since it was possible that the RT-PCR of *sigQ* may not have worked because *sigQ* is not expressed at 24 hours during development, first-strand cDNA was synthesized from the M600 (48 hour) RNA sample and the *ΔbldD* FLP 49/pAU244 (120 minute) RNA sample. Recall that *sigQ* expression had been previously shown to be activated (~20 fold) upon *bldD* induction (C. Galibois, unpublished). End-point PCR (28 cycles) was performed using the RT reactions as template. Again, intense hrdBF-hrdBR product was detected (Figure 3.24A). The LB5-LB6 product, although much fainter, was seen in the M600 (48 hour) sample. Unexpectedly, no LB5-LB6 product was seen for the *ΔbldD* FLP 49/pAU244 (120 minute) sample. This was a surprising result because it was expected that *sigQ* would be activated when BldD was overexpressed.

The faintness in the M600 samples of *sigQ* signal compared to *hrdB* signal could be due to the presence of fewer *sigQ* transcripts than *hrdB* transcripts in the entire RNA population. Alternatively, cDNA synthesis with LB6 primer may not be as efficient as with hrdBR primer. First-strand cDNA synthesis for the microarray analyses performed by C. Galibois had used random hexamers. Therefore, to explore the possibility that the LB6 primer was inefficient for cDNA synthesis, it was decided that random hexamers, in place of gene-specific primers, would be used to prime cDNA synthesis. The use of random hexamers would give the whole cDNA population. First-strand cDNA was synthesized from the M600 (15 hour) and M600 (48 hour) RNA samples and end-point PCR was performed with the resulting RT reactions. This time, the amounts of LB5-LB6 product and hrdBF-hrdBR product were more comparable (Figure 3.24B) so a decision

(A) Gene-specific primers to prime cDNA synthesis


M600 DNA
M600 (48 h) + SSIII
M600 (48 h) - SSIII
ΔbldD FLP 49/pAU244 (120 min) + SSIII
ΔbldD FLP 49/pAU244 (120 min) - SSIII
M600 DNA
M600 (48 h) + SSIII
ΔbldD FLP 49/pAU244 (120 min) + SSIII
125 bp
101 bp
LB5-LB6 product
hrdBF-hrdBR product

(B) Random hexamers to prime cDNA synthesis

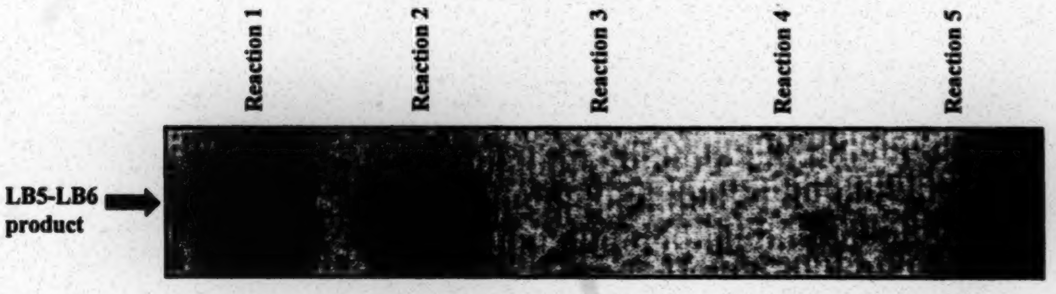

M600 (15 h) + SSIII
M600 (15 h) - SSIII
M600 (48 h) + SSIII
M600 (48 h) - SSIII
M600 DNA
M600 (15 h) + SSIII
M600 (15 h) - SSIII
M600 (48 h) + SSIII
M600 (48 h) - SSIII
M600 DNA
125 bp
101 bp
LB5-LB6 product
hrdBF-hrdBR product

was made to go ahead with real-time RT-PCR experiments.

The real-time RT-PCR experiments are similar to end-point RT-PCR experiments except that the former technique is more accurate because it monitors the progress of the PCR rather than the amount of accumulated PCR product at the end of the PCR cycle. In the real-time PCR method, the cDNA sample is used as template in a PCR reaction containing SYBR® Green I, a fluorescent dye that binds double-stranded DNA. Therefore, as PCR product is produced, it is bound by SYBR® Green dye. The higher the starting number of cDNA species containing *sigQ* gene sequence, the sooner amplification of *sigQ* PCR product occurs, and therefore the sooner significant fluorescence is observed. Even small differences in transcript levels between samples can be detected using the real-time method whereas the end-point method provides meaningful results only if there are significant differences in transcript levels between samples.

Before real-time PCR analyses could be carried out, a validation experiment had to be performed to test the PCR efficiencies of the primer pairs to be used for amplification of the test and control genes. This was necessary to determine which method of relative quantification would be most appropriate to quantitate the results of the real-time RT-PCR analyses. If the amplification efficiencies of the two primer pairs are the same, then the comparative C_T method would be used to quantitate the results. If the amplification efficiencies of the two sets of primers are different, the standard curve method must be used to quantitate the real-time PCR results. The former quantitation method is preferred because it offers the advantage of increased throughput.

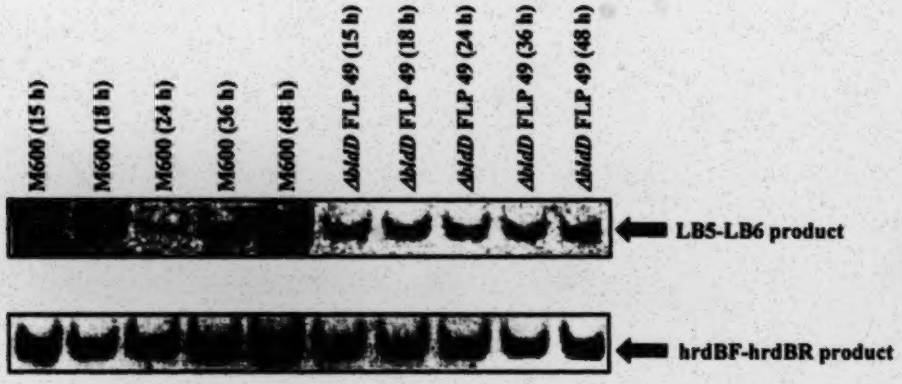
The first and second attempts to perform the validation experiments were unsuccessful. In both cases, *hrdB* signal was detected at 17 cycles while *sigQ* signal could barely be detected even at 35 cycles, and since a large number of cycles was required before product was seen, the resulting product was most likely due to genomic DNA contamination. To try and address the failure to detect *sigQ*-specific signal, the real-time buffer conditions were tested. To do this, cDNA was synthesized using random hexamers and the RT reaction was used in end-point PCR reactions. It was found that the LB5-LB6 product could be detected when PCR reactions used either in-house Taq polymerase (provided by M. Pickard, Department of Biological Sciences, University of Alberta), or the commercial Platinum® Taq polymerase with Taq buffer [50 mM Tris-Cl, pH 9.0, 1.5 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, 0.1 mg/mL BSA (non-acetylated), 10 mM NH_4SO_4, 0.2 mM dNTPs] containing 1 M betaine, while other real-time buffers (ABI, Platinum®, Power) did not work (Figure 3.25). While the components of the ABI (from Applied Biosystems) and Power (a newer version of the ABI buffer which is also supplied by Applied Biosystems) buffers are unknown because they are proprietary, the Platinum® buffer, which was provided by T. Locke (MBSU, Department of Biological Sciences, University of Alberta) contains a final reaction concentration of 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.8% glycerol (v/v), 0.01% Tween 20 (v/v), 2% DMSO (v/v), 0.2 mM dNTPs, 1X ROX (Invitrogen), 0.25X SYBR® Green I dye (Invitrogen), and 0.03 units/μL Platinum® Taq polymerase (Invitrogen). Aside from the SYBR® Green I dye and ROX reference dye, the differences between this buffer and the Taq buffer (described above) is probably negligible because each is optimized for a different Taq polymerase. On the other hand, the SYBR® or ROX present in the


Reaction 1
Reaction 2
Reaction 3
Reaction 4
Reaction 5
LB5-LB6
product

Platinum® buffer might somehow be inhibitory for the LB5-LB6 PCR. However, addition of these components to the Taq buffer resulted in detection of LB5-LB6 product using either Taq polymerase or the commercial Platinum® Taq polymerase (data not shown). Since the *sigQ* product was amplified in the end-point PCR reactions, the real-time PCR validation experiments were performed with the new optimized conditions, but again no *sigQ* product was generated until after 35 cycles (data not shown).

Since *sigQ*-specific signal could not be detected under the real-time PCR conditions, it was decided that semi-quantitative (20-22 cycles) end-point RT-PCR would be used instead to compare *sigQ* expression in the *S. coelicolor* wild-type strain to the *ΔbldD* FLP 49 strain and in the BldD overexpression strain to the vector control strain. First-strand cDNA was synthesized from all RNA samples from the solid medium time course (described above) and then the *sigQ* product was amplified using the LB5-LB6 primer pair. As a control for RNA levels, *hrdB* product was also amplified from the same cDNA sample using the hrdBF-hrdBR primer pair. Using 22 cycles for the PCR amplification, differences were seen in *sigQ* expression between the wild-type and *ΔbldD* FLP 49 strains (Figure 3.26A). Significantly higher levels of LB5-LB6 product were amplified from the 15 hour and 48 hour time point samples. However, some of the signal can be attributed to higher RNA loads in those reactions as demonstrated by the higher levels of *hrdB* product amplified. Despite the RNA loading differences, the *sigQ* signals do appear to be significantly higher in some of the M600 samples when compared to the *ΔbldD* mutant. Therefore, these results not only suggest that *sigQ* exhibits biphasic expression, peaking in early vegetative growth and then again during sporulation, but that BldD does activate its transcription during those time points in colony growth. Since

(A) Solid medium time course


M600 (15 h)
M600 (18 h)
M600 (24 h)
M600 (36 h)
M600 (48 h)
ΔbldD FLP 49 (15 h)
ΔbldD FLP 49 (18 h)
ΔbldD FLP 49 (24 h)
ΔbldD FLP 49 (36 h)
ΔbldD FLP 49 (48 h)
LB5-LB6 product
hrdBF-hrdBR product

(B) Liquid medium induction time course

1169/pIJ6902 (0 min)
1169/pIJ6902 (15 min)
1169/pIJ6902 (30 min)
1169/pIJ6902 (45 min)
1169/pIJ6902 (60 min)
1169/pIJ6902 (120 min)
1169/pAU244 (0 min)
1169/pAU244 (15 min)
1169/pAU244 (30 min)
1169/pAU244 (45 min)
1169/pAU244 (60 min)
1169/pAU244 (120 min)
LB5-LB6 product
hrdBF-hrdBR product

there was some doubt about the level of BldD induction in the liquid medium time course (described above), as a control for the semi-quantitative RT-PCR, cDNA was instead synthesized from RNA samples isolated from the liquid medium induction time course previously used in microarray analysis (C. Galibois, unpublished). It should be noted that this RNA was isolated in 2001 and has since then been stored at -86°C. RNA from this time course had been harvested at various time points post-inoculation from the *S. coelicolor* 1169 *bldD* point mutant harbouring either pAU244 or the parent pIJ6902 plasmid. As stated above, the microarray analysis of these RNA samples showed *sigQ* activation following BldD overexpression. Using 20 cycles for the PCR amplification after cDNA synthesis, *sigQ* expression appeared to be induced when BldD was overexpressed (Figure 3.26B). The LB5-LB6 product was more intense in all time points (except for the 45 minute sample in which the *sigQ* transcript may now be degraded) of the overexpression strain compared to that of the vector control strain and that of the 0 minute time point (uninduced sample) of the overexpression strain. Although there was no time to repeat these experiments, both the solid culture and liquid culture results do suggest that *sigQ* expression is activated by BldD.

3.4. Phenotype of *ΔsigQ* null mutant

In order to identify a possible role for BldD in morphological differentiation and antibiotic synthesis, it was necessary to construct a *ΔsigQ* null mutant and then assess its phenotype for any visible defects in differentiation or antibiotic production. A *ΔsigQ* null mutant was previously constructed prior to the commencement of this thesis work (L. Bui, summer 2003 project work). Construction of the *ΔsigQ* null strain used the REDIRECT© technology (Gust et al. 2003) and proper construction of this mutant strain was verified by PCR and Southern hybridization analysis (data not shown). To identify a possible role for *sigQ* in differentiation or antibiotic production, the *ΔsigQ* null mutant was grown alongside the wild-type M600 strain on various *Streptomyces* media, each containing a different complement of nutrients (all media described by Kieser et al. 2000) and the phenotype of the two strains was compared. Disappointingly, the *ΔsigQ* null mutant was observed to have an identical phenotype to that of the wild-type strain when grown for five days on ISP4 (international *Streptomyces* project 4), MS (mannitol-soy), R2YE (sucrose yeast extract), SMMS (supplemented minimal medium, solid), and MM (minimal medium) + glucose, and MM + mannitol (data not shown). Since *sigH*, another BldD target, has been shown to be involved in osmotic stress (Kelemen et al. 2001; Viollier et al. 2003), it was decided to assess a potential role for *sigQ* in osmotic stress. To do this, the *ΔsigQ* mutant was plated alongside the wild-type strain on MM + glucose, MM + glucose + 0.5 M NaCl, MM + glucose + 1 M NaCl, and MM + glucose + 1 M sucrose. The phenotype of the two strains was observed every 24 hours for four days and no differences in phenotype were observed between the two strains. The role of the *sigQ*-encoded sigma factor therefore still awaits discovery. Knowledge of the function of this sigma factor may give clues about other genes that might also be activated by BldD.

CHAPTER 4:

DISCUSSION

4. DISCUSSION

Study of the BldD protein is of great importance because this protein is a transcriptional regulator playing a critical role in controlling morphological differentiation and antibiotic synthesis in *S. coelicolor*, although little is known of how BldD exerts its control on the latter process. Prior to the initiation of this thesis work, much work had been performed by M. Elliot to characterize the mechanism by which BldD exerts its control on morphological differentiation in *S. coelicolor*. It was observed that during vegetative growth, BldD represses the expression of three sigma factor genes including *bldN*, required for initiation of aerial mycelium formation, *whiG*, needed for initiation of sporulation (Elliot et al. 2001), and *sigH*, important for differentiation and osmotic stress (Kelemen et al. 2001). Since sigma factors play an important role in the transcription of genes by conferring promoter specificity to RNA polymerase, it can be concluded that through the control of *bldN*, *whiG*, and *sigH* expression, BldD probably indirectly regulates the expression of a number of other genes important for the process of differentiation. In addition to these three genes, BldD also negatively regulates the expression of *bdtA*, encoding a protein of unknown function (Elliot et al. 2001).

The studies described above provided strong evidence for the role of BldD as a repressor of gene expression. However, it has long been hypothesized that in addition to its role as a repressor, BldD likely also functions to activate transcription of genes involved in morphological differentiation and antibiotic synthesis (B. Leskiw, personal communication; Elliot et al. 2001). This hypothesis was based on the observation that a *bldD* mutant is defective in both antibiotic production and aerial mycelium formation, a phenotype that is not expected because the absence of a negative regulator of these processes should give a phenotype of accelerated development and precocious antibiotic synthesis. It is possible, however, that in addition to repressing positive regulators of development and antibiotic production, BldD also functions to repress negative regulators of these processes. In this case, the absence of BldD would result in prolonged expression of these negative regulators, which might then cause the observed pleiotropic defects in differentiation and antibiotic synthesis of the *bldD* mutant. Another possibility is that the mutant phenotype is simply a manifestation of the premature expression of developmental and antibiotic synthesis genes, which would disrupt the organism's regulatory circuit and lead to detrimental effects. Support for the idea that BldD also functions as a transcriptional activator was obtained when microarray analysis examining global gene expression in *S. coelicolor* following *bldD* induction identified a putative BldD target whose expression was activated when the BldD protein was overexpressed (C. Galibois, unpublished). DNA-binding studies (electrophoretic mobility shift assays) confirmed that BldD could bind to the promoter region of this newly-identified gene target. Interestingly, this gene, designated *sigQ*, encodes another sigma factor whose function is not yet known. The regulation of sigma factors appears to be a common theme for BldD, as it has already been shown that BldD controls the expression of three other sigma factor genes. It can be said that BldD plays a key role in the control of gene expression in *S. coelicolor* by functioning as a regulator of regulators.

Analysis of the BldD sequence using the PFAM search indicated that the N-terminal domain of BldD exhibits significant similarity to the HTH-3 DNA-binding domain of proteins belonging to the XRE (xenobiotic response element) family of transcriptional regulators (Kelemen et al. 2001). Very recent elucidation of the crystal structure of the N-terminal DNA-binding domain of BldD revealed that the three-dimensional (3D) structure of the DNA-binding domain of BldD is very similar to the N-terminal DNA-binding domain of the bacteriophage lambda CI protein (lambda repressor), another member of the XRE family of transcriptional regulators (Kim et al. 2006). This finding is interesting because the lambda repressor is known to function as both a repressor and an activator of gene transcription (Ptashne 1992). To maintain life as a lysogen in *E. coli*, the bacteriophage lambda CI protein binds to DNA operator regions on the phage genome to prevent transcription of phage genes needed for lytic growth but at the same time it activates transcription of the *cI* gene, which codes for the lambda repressor, itself. Therefore, as is the case with lambda repressor, it is possible that BldD represses expression of developmental genes during vegetative growth but at the onset of differentiation, BldD derepression occurs simultaneously with BldD activation of some of the same, or perhaps different, developmental genes. There is already evidence to suggest that BldD, although repressing the transcription of *whiG* during vegetative growth, might also activate transcription of the same gene during sporulation (Elliot et al. 2001). This idea is based on the observation that *whiG* transcript levels are much higher in a *bldD* mutant strain compared to that in the wild-type strain during vegetative and aerial growth; however, during sporulation, *whiG* transcripts are virtually absent in the *bldD* mutant. The mechanism(s) involved in controlling the switch between the BldD repressor and activator functions, if this process is occurring, is unknown; although it has been previously postulated that post-translational mechanisms may play a role (Elliot 2000; Elliot et al. 2001).

Post-translational mechanisms such as phosphorylation, proteolytic processing, and protein-protein interactions are often used to modify, and thus, control the activity of transcriptional regulators. Based on the finding that the mutated *bldD* gene contains an A to G substitution at position 62 resulting in a tyrosine to cysteine change, it was initially hypothesized that BldD might be modified by phosphorylation at this tyrosine residue (Elliot 2000; Elliot et al. 2001). However, Western analysis using antibodies specifically raised against phosphorylated tyrosine revealed that this tyrosine residue is likely not phosphorylated (Elliot 2000). More recently, the crystal structure of the BldD N-terminal DNA-binding domain revealed that the side chain of Tyr62 is directed at the center of the globular domain of the BldD N-terminus, suggesting that this amino acid residue is important for maintaining the hydrophobic core of BldD (Kim et al. 2006). Mutation of this residue to either cysteine or serine, both of which are small amino acids, led to core destabilization and loss of BldD function while mutation to phenylalanine, another hydrophobic amino acid, had no visible effects on BldD function.

Also included in the XRE family of transcriptional regulators is SinR, a *B. subtilis* repressor protein involved in regulating many important processes such as sporulation and competence in this organism (Gaur et al. 1991). SinR derepression is achieved when SinR is bound by its antagonist, SinI, which ultimately abolishes the DNA-binding activity of SinR (Bai et al. 1993). Like SinR, BldD activity may also be altered when bound to a protein partner. The identification of any BldD-binding partner(s) would then facilitate understanding of the regulation of BldD activity.

Two-dimensional PAGE analysis of wild-type *S. coelicolor* cellular extract proteins by A. Hesketh (personal communication) identified a BldD isoform, potentially a modified form of BldD. This probable modified BldD protein is slightly more basic than unmodified BldD and is estimated to be ~1 kDa larger than unmodified BldD. This finding suggests that besides protein-protein interaction, specific modification of BldD protein might also be a mechanism to modulate its activity. A. Hesketh observed that modified BldD is absent during mid-exponential phase growth and begins to accumulate at late-exponential phase growth and reaches higher levels as the organism enters stationary phase growth. In contrast, unmodified BldD is present at high amounts during mid-exponential phase growth and begins to decline in amounts as the organism progresses into late-exponential and stationary phase growth. Although decreasing in protein levels, the unmodified BldD protein is present at all time points in growth, suggesting that BldD modification cannot be the only mechanism involved in overcoming BldD-mediated transcriptional repression of target genes. One possibility is that one mechanism might be involved in relieving BldD repression of gene expression while a second mechanism might operate to modify BldD to an activator role.

The objective of this thesis was to explore further the possible post-translational mechanisms involved in regulating BldD activity. One main focus of the thesis was to extend the Hesketh 2D-PAGE study by attempting to isolate and characterize the observed BldD isoform that accumulates with culture age. The second main focus was to attempt to isolate and characterize any BldD-binding partner(s) that might be needed either for overcoming BldD repression or for directing the BldD activator role.

Several techniques including 2D-PAGE, immunoprecipitation, and DNA affinity chromatography were explored to isolate the BldD isoform previously observed in the Hesketh study. It was hoped that following isolation of the BldD isoform, mass spectrometry could be employed to characterize the modification, if it exists. Based on the work by A. Hesketh, it was expected that the two-dimensional PAGE technique would facilitate successful separation of the unmodified and modified BldD proteins. However, unexpectedly, when proteins present in *S. coelicolor* cell-free extracts were resolved with 7 cm isoelectric focusing strips and small format second dimension gels, Western analysis detected at least five major BldD-related spots instead of the two spots seen by A. Hesketh. However, since A. Hesketh never performed Western analyses but rather identified the BldD spots after random spot picking and mass spectrometric analyses of proteins separated by 2D-PAGE, it is not known if additional BldD spots were present on his gels and simply not picked and identified. Since the 2D-PAGE standards available and used in this thesis work were not consistently detectable by Coomassie Brilliant Blue staining, it was not possible to use pI value along with molecular weight to estimate the locations of the BldD isoforms. Also, since characterization of the BldD isoforms requires visualization of the protein spots on the gel following Coomassie staining, so that observed spots can be excised and subjected to mass spectrometric analysis, it was decided that 2D spot patterns would be compared between wild-type *S. coelicolor* and a *ΔbldD* mutant. In the absence of standard marker proteins on the gel, the identification of any spots migrating in the BldD molecular weight range that are present in the wild-type cell-free extract but absent from the *ΔbldD* mutant cell-free extract could serve to identify protein spots of interest. To increase the chances of detecting the BldD isoforms, efforts were made to resolve larger amounts of total protein content (400 μg instead of 100 μg) using 7 cm isoelectric focusing strips and small format second dimension gels, but this resulted in poor isoelectric focusing, most likely caused by protein overloading of the isoelectric focusing strips. Efforts to resolve larger amounts of total protein content using 18 cm isoelectric focusing strips and larger format second dimension gels were more promising but the method was abandoned because there was no time to optimize the technique for detection of the BldD isoforms. If time had permitted, it would have been ideal to optimize the larger format 2D-PAGE technique. Once optimized, 2D spot patterns could be compared between wild-type *S. coelicolor* and a *ΔbldD* mutant to determine which spots might correspond to the BldD isoforms. Replicate separations of proteins in wild-type *S. coelicolor* cell-free extract could then be performed and subsequent gel staining with Sypro® Ruby, a protein stain that is more sensitive than Coomassie Brilliant Blue, might reveal spots corresponding to the BldD isoforms, which could then be excised from the replicate gels and pooled before mass spectrometric analysis.

As an alternative to the 2D-PAGE technique, immunoprecipitation was pursued. Although this technique led to the isolation of unmodified BldD protein, the putative modified BldD protein was not identified in the mass spectrometric analyses that were performed. A likely reason for the failure of this technique was that the small difference in molecular weight sizes of the BldD isoforms (estimated to be ~1 kDa based on the Hesketh study) did not allow for one-dimensional separation of the isoforms. Therefore the protein band that was sent for mass spectrometric characterization was expected to contain both unmodified and modified BldD protein. Although the mass spectrometric technique is very sensitive, the very low amount of modified BldD, if it even existed, could not be detected even when IP experiments were scaled up so that much more IP sample could be subjected to mass spectrometric analysis. Tryptic digestion of a mixture of proteins in which there was an excess of unmodified BldD would be expected to generate mostly peptides belonging to the unmodified protein. The end result would be that any low abundance modified peptides, if present, would be masked by the more abundant unmodified peptides. To try and address this problem, a combination of IP and 2D-PAGE were performed as a way to facilitate purification of the modified BldD protein so that this protein species alone could be subjected to mass spectrometric analysis. However, the overwhelming amount of antibody contamination of the IP samples hindered proper isoelectric focusing of the IP samples and therefore the modified BldD protein was not separated from the unmodified BldD protein following 2D-PAGE. Coupling of the antibodies to protein A Sepharose was attempted to reduce the antibody contamination of the IP samples but the method used for the coupling did not lead to efficient irreversible coupling and the combined method had to be abandoned. If the antibody contamination problem could have been solved, the combined IP and 2D-PAGE approach may have resulted in information about the proposed BldD modification.

The next approach to isolate the BldD isoforms involved using DNA affinity chromatography with the expectation that BldD would bind to the *bldD* promoter region in its repressor, likely unmodified, form while possibly binding to the *sigQ* promoter region in its activator, perhaps modified, form. It was hoped that differences between the two bound protein forms could then be characterized by mass spectrometric analysis. Although after much optimization, this technique finally led to the purification of unmodified BldD from *S. coelicolor* cell-free extracts using the *bldD* promoter fragment as bait, neither unmodified nor modified BldD protein could be isolated using the *sigQ* promoter fragment as bait. This finding suggested that the binding of BldD to the *sigQ* promoter region to activate its transcription might require a cofactor protein. One possibility is that BldD binding to the *sigQ* promoter region occurs only at a very specific time during growth, under very specific conditions when the ratio of BldD and its cofactor protein is ideal. However, this hypothesis would be refuted by the results of previous *in vitro* DNA-binding assays that revealed that purified His$_6$-BldD protein (expressed in *E. coli*) could bind to the *sigQ* promoter fragment (C. Galibois, unpublished). At any rate, if the binding of BldD to the *sigQ* promoter requires an interacting protein partner and binding is transient, the use of chemical crosslinkers to stabilize the BldD-binding partner complex might facilitate the capture of modified BldD with the *sigQ* promoter fragment as bait. In addition, the use of cell-free extracts over a time course might eventually lead to affinity purification of modified BldD protein. Finally, successful affinity purification of modified BldD might require scale-ups of culture volumes because modified BldD likely is present at very low amounts, as is the usual case for modified proteins (Seo and Lee 2004).

The next part of the thesis involved attempts to isolate and characterize potential BldD-binding partners. For these studies a system for overexpression of His$_6$-BldD in *S. coelicolor* was created so that nickel affinity chromatography could then be used for efficient, one step, purification of His$_6$-BldD and any bound protein partner(s). To ensure the capture of BldD-binding partners, which might only bind to BldD transiently, chemical crosslinking would then be performed to stabilize the interaction. After much effort, a His$_6$-BldD overexpression system was finally constructed; however, purification of the His$_6$-BldD was never successful, and therefore another technique to isolate BldD-binding partners was pursued. Since immunoprecipitation had been successful in isolation of BldD monomer, it was decided to use this technique again to purify BldD-dependent complexes, if they existed. Western analysis detected a single BldD complex following *in vitro* chemical crosslinking of cellular extract proteins isolated from a BldD overexpression strain but for unknown reasons this complex could not be seen following immunoprecipitation and detection of the IP sample by Coomassie staining.

Since the biochemical studies performed to characterize BldD post-translational modification were repeatedly unsuccessful, the decision was made to direct the attention of the thesis to strengthening the evidence that supported the idea that BldD activates *sigQ* expression. To do this, it was necessary to confirm that *sigQ*, the only known putative activated BldD target, is in fact a BldD target. As mentioned above, previous analysis of BldD binding to the *sigQ* promoter had involved the use of an *in vitro* DNA binding assay (C. Galibois, unpublished) so in order to authenticate those results, BldD binding to the *sigQ* promoter sequence was tested using an *in vivo* methodology. The method chosen was chromatin immunoprecipitation (ChIP). The ChIP analyses did confirm BldD binding to the *sigQ* promoter but they suggested that *sigQ* was only a weak BldD target. This was an unexpected result because the *sigQ* promoter region contains many putative BldD binding sites (17 half sites). Again, it is possible that the ChIP results showed poor BldD binding to the *sigQ* promoter because protein-DNA crosslinking had been performed with cultures where the ratio of BldD to its cofactor protein was not ideal. To try to gain a better sense of whether or not this was true, real-time RT-PCR experiments were performed both to confirm BldD dependence of *sigQ* expression and to address the timing of maximal *sigQ* expression. Despite many attempts to optimize the real-time RT-PCR technique, *sigQ*-specific signal could never be detected and therefore this technique was abandoned. Semi-quantitative RT-PCR, however, did support the initial microarray analyses that showed that *sigQ* expression is activated by BldD. Interestingly, *sigQ* appears to exhibit a biphasic expression pattern with peak expression occurring at time points corresponding to vegetative growth and sporulation, and almost negligible expression at the time point corresponding to the onset of aerial mycelium formation. This result suggests that during vegetative growth and sporulation BldD activates *sigQ* expression, but at the onset of aerial mycelium formation BldD represses expression of *sigQ*. Since there seems to be differential *sigQ* expression during growth, this could explain why the ChIP results reflected such poor binding of BldD to the *sigQ* promoter. However, the RT-PCR experiment described above was performed with RNA isolated from surface-grown cultures while ChIP experiments used liquid-grown cultures so it is difficult to correlate the pattern of *sigQ* expression in surface cultures with that in liquid cultures. Although a control semi-quantitative RT-PCR analysis of RNA isolated from the *bldD* point mutant harbouring the pAU244 plasmid for inducible expression of BldD confirmed that *sigQ* is activated when BldD is overexpressed, strong conclusions cannot be made from the results of the semi-quantitative RT-PCR analyses because these experiments were performed only once. Moreover, results from end-point RT-PCR analysis are not as accurate as those that are obtained from real-time RT-PCR analysis. If time had permitted, it would have been ideal to optimize the real-time technique. In this study, only one primer set was used to amplify *sigQ* from the cDNA population and it was never addressed whether different primer sets would have allowed for *sigQ*-specific signal under the real-time PCR conditions. Nevertheless, since the semi-quantitative RT-PCR results from the BldD overexpression strain showed significant *sigQ* activation following *bldD* induction, an interesting future experiment would be to revisit the ChIP technique to examine whether DNAs containing the *sigQ* promoter region would be more highly enriched when BldD is overexpressed. Furthermore, since BldD may require a binding partner to interact with the *sigQ* promoter, the use of protein crosslinkers, in addition to formaldehyde, in the protein-DNA crosslinking reaction might result in enrichment of DNAs containing the *sigQ* promoter sequence following BldD immunoprecipitation. Other researchers have found that the use of a protein-protein crosslinker together with formaldehyde facilitated more reproducible enrichment of target DNA, presumably because many proteins function within larger protein complexes and the use of a protein-protein crosslinker would help stabilize these protein complexes (Kurdistani and Grunstein 2003; Nowak et al. 2005).

In this study, interpretation of the DNA affinity chromatography and ChIP results was based on the assumption that BldD would bind to the *sigQ* promoter with high affinity because this DNA region contains many putative BldD binding sites; however, it is not known whether BldD actually binds to all the repeat sequences within the *sigQ* promoter. Although *in vitro* DNA binding studies showed that BldD could bind with similar affinity to *sigQ* promoter fragments containing either the upstream half of the repeat sequences (9 repeats) or the downstream half of the repeat sequences (8 repeats) (C. Galibois, unpublished), further work has not been performed to clearly define the repeat sequences bound by BldD. It is possible that BldD binds only to certain repeats in the *sigQ* promoter, and this binding somehow induces a bend in the DNA that is favourable for enhanced binding of RNA polymerase holoenzyme to the *sigQ* promoter, thus causing activation of *sigQ* transcription. This mechanism of transcriptional regulation could possibly account for the observed weak affinity binding of BldD to the *sigQ* promoter. Therefore future work to define the BldD binding site(s) within the *sigQ* promoter is needed. To define the BldD binding site, either DNase I footprinting or hydroxyl radical footprinting may be employed, with the latter technique being more sensitive.

Finally, the phenotype of a *ΔsigQ* null mutant was examined to assess the role of *sigQ* in morphological differentiation and/or antibiotic synthesis and/or osmotic stress. A clear role for *sigQ* in these processes could not be assigned because the *ΔsigQ* null mutant was observed to have the same phenotype as the wild-type M600 strain with respect to differentiation, antibiotic production, and osmotic tolerance. Although it was disappointing that a clear role for *sigQ* in these processes could not be defined based on phenotypic studies, it is certainly not surprising that the disruption of a sigma factor gene did not result in a mutant phenotype since the *S. coelicolor* genome codes for some 65 sigma factors, and sigma factor redundancy has been observed (Viollier et al. 2003). Disruption of the *sigH* gene, encoding the osmosensitive sigma factor, σ^H, did not produce a mutant phenotype with respect to salt tolerance, and this was likely due to the presence of multiple paralogous sigma factors (σ^I and σ^J) that function with σ^H to regulate the response to osmotic stress (Viollier et al. 2003). Clearly, the function of the *sigQ*-encoded sigma factor needs further investigation. It has been speculated that *sigQ* might play some role in antibiotic production because it is located near *afsQ1* and *afsQ2*, which encode a two-component system involved in antibiotic production. While disruption of the *S. coelicolor afsQ1* and *afsQ2* genes did not cause any visible defects in antibiotic production, introduction of these genes, on a multi-copy plasmid, into *S. lividans* stimulates production of the *S. coelicolor* pigmented antibiotics (Ishizuka et al. 1992). It would be interesting to see if there are any regulatory connections between *sigQ* and the *afsQ1-afsQ2* loci. Further clues about the role of *sigQ* could also be achieved from transcriptional analysis of *sigQ* under various stresses or in different developmental mutants. Knowledge about the function of the *sigQ* product might provide understanding of why BldD activates *sigQ* transcription and could therefore lead to the discovery of other genes that are activated by BldD.

Overall, the various biochemical techniques explored in this study did not lead to further understanding of post-translational control of BldD. While these techniques allowed for the isolation of unmodified BldD protein, isolation of modified BldD protein(s) was repeatedly unsuccessful and therefore, characterization of the putative BldD modification(s) was impossible. Isolation of potential BldD-binding partners was also unsuccessful. However, the work on *sigQ* did suggest that *sigQ* is an activated BldD target, although the role of the *sigQ* product is still unknown.

There are certainly other avenues of research that might lead to further understanding of post-translational control of BldD. The possibility of involvement of proteolytic processing in post-translational control of BldD was not explored in this thesis. Kim et al. (2006) speculate that the activity of BldD protein might be modified through proteolytic cleavage because repeated attempts to crystallize full-length BldD protein had been unsuccessful. This hypothesis is supported by the observation that the 3D structure of BldD is very similar to the lambda repressor, which is regulated by RecA-mediated self-cleavage between the N- and C-terminal domains of the protein (Kim and

Little 1993; Roberts and Roberts 1975). Although self-cleavage of BldD probably does not occur because BldD does not appear to have a self-cleavage site within its sequence, BldD cleavage may be mediated by a general protease that is somehow triggered to act on BldD at the onset of differentiation. It has already been observed in the course of this thesis that BldD protein levels begin to decrease during the late stages of aerial growth and levels are barely detectable during sporulation. On the other hand, BldD protein levels are relatively constant in submerged culture, conditions where *S. coelicolor* does not undergo differentiation. Therefore, degradation of BldD might be important for differentiation. There is evidence that suggests that Clp-mediated proteolysis is important for *Streptomyces* development (de Crecy-Lagard et al. 1999; Viala and Mazodier 2003). It is attractive to speculate that the Clp protease exerts its control on differentiation by degrading BldD, which then results in derepression of developmental genes. Certainly, there is the possibility that another general protease is involved in BldD cleavage. A possible future experiment could involve monitoring BldD degradation in various protease-deficient backgrounds to identify the protease involved in BldD processing. It should be noted, however, that proteolysis is probably not the only mechanism involved in regulating BldD activity. It is unlikely that this mechanism is involved in regulating the initiation of aerial mycelium formation because BldD levels are similar at time points corresponding to vegetative growth (15 hours), the transition between vegetative and aerial growth (18 hours), and aerial growth (24 hours).

It might also be worthwhile to re-examine the involvement of phosphorylation in regulating BldD activity. Although the tyrosine residue at position 62 appears to function in maintaining BldD core stability rather than functioning as a target for phosphorylation

(Kim et al. 2006), the possibility of phosphorylation at other amino acid residues (serine and threonine) still remains. The 2D-PAGE analyses performed in this thesis consistently revealed three BldD isoforms with different pI values but similar molecular weights, which is a characteristic feature of phosphorylation.

Although proteolytic cleavage or phosphorylation are valid mechanisms for regulating BldD activity, recall that the putative modified BldD protein observed by A. Hesketh was estimated to be ~1 kDa larger than unmodified BldD, meaning that it cannot be a product of proteolytic cleavage or phosphorylation (80 Da). While most other modifications result in a modified protein that is only slightly larger than its unmodified counterpart, protein glycosylation usually causes a size change >800 Da (Seo and Lee 2004), which is consistent with the molecular weight difference between the BldD isoforms observed by A. Hesketh. Alternatively, the observed size increase of modified BldD could be caused by a peptide addition to BldD, which might direct BldD for proteolysis. In *E. coli*, translation is regulated by a tmRNA tagging system, which involves the addition of a peptide (AANDENYALAA) to the C-terminus of proteins following the stalling of ribosomes during translation (reviewed by Withey and Friedman 2003). Proteins containing the tagged peptide are then recognized and degraded by ATP-dependent proteases such as ClpP or FtsH. Since the approximate molecular weight of an amino acid is 100 Da, the tmRNA-dependent peptide tag of 11 amino acids would give a molecular weight size (~1 kDa) that is consistent with the observed molecular weight increase of modified BldD. Although there is evidence to indicate that a tmRNA tagging system exists and functions in *Streptomyces*, it appears so far that this system is not important for *Streptomyces* differentiation (Braud et al. 2006). Nevertheless, it would be interesting to examine whether regulation of BldD activity involves proteolysis mediated by the tmRNA tagging system.

Further work to identify a BldD-binding partner is necessary. Modulation of the activity of some dual regulators belonging to the MerR and AraC family of regulators requires binding with either small effector molecules or protein ligands, respectively (Brown 2003; Plano 2004). Although biochemical methods were used in this study in hopes of identifying a BldD-binding partner, certainly, there are genetic methods available to study protein-protein interactions. One powerful genetic method to study *in vivo* protein-protein interaction is the bacterial two-hybrid system, although this system would need to be optimized for work in *Streptomyces*. Furthermore, since there are no predicted BldD-binding partners, the use of the bacterial two-hybrid system would involve screening of the entire genome for genes encoding potential BldD-binding partners. This is a difficult feat and the chance of success is minimal.

Clearly, more work is needed in establishing the involvement of post-translational regulation of BldD activity. Although this area of research is proving to be very difficult, understanding the regulation of BldD activity is important because BldD is a key regulator. In addition to regulating the four sigma factors mentioned in this thesis, there is also evidence that BldD regulates yet another sigma factor (C. Galibois, unpublished). A clearer understanding of how BldD exerts its effects on all these different regulators is necessary before we can begin to understand fully its role in the onset of morphological differentiation and antibiotic biosynthesis.

CHAPTER 5:

REFERENCES

5. REFERENCES

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